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H. R. Brown	Boston	Theobald Smith	1904-1905
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By Wilfred H. Manwaring. (*Aus dem Kgl. preuss. Institut für Infektionskrankheiten, Berlin.*)
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## THE USE OF THE FERMENTATION TUBE IN INTESTINAL BACTERIOLOGY.

By C. A. HERTER, MD., AND A. I. KENDALL, PH.D.

(Received for publication, July 5, 1908.)

During the progress of a series of investigations bearing upon the bacterial flora of the intestinal tract of infants and adults in health and disease, the writers have obtained valuable information from the routine use of the fermentation tube, by using methods similar to those of Dr. Theobald Smith,<sup>1</sup> who has repeatedly called attention to the fundamental importance of this apparatus in the study of fermentative bacteria. His researches have shown conclusively that the gas volume, gas ratio (proportion of gas soluble in caustic alkali to the insoluble portion) and the length of time necessary to produce the maximum amount of gas are characteristics of prime importance in the study of this group of organisms. Furthermore, by the simple addition of bits of fresh sterile animal tissue<sup>2</sup> he has succeeded in cultivating in these tubes a number of anaërobes which would not grow under ordinary conditions.<sup>3</sup> Finally, by introducing the use of milk in

<sup>1</sup> Das Gährungskölbchen in der Bakteriologie, *Centralbl. f. Bakt.*, vii, pp. 502-506, 1890; Einige Bemerkungen über Säure- und Alkalibildung bei Bakterien, *Ibid.*, viii, p. 389, 1890; The Fermentation Tube, etc., *Wilder Quarter-Century Book*, pp. 187-234, 1893.

<sup>2</sup> *Centralbl. f. Bakt.*, vii, pp. 502-506, 1890.

<sup>3</sup> There seems to be considerable question about the priority of this procedure. According to Marino (*Méthod pour isoler les anaërobies*, *Ann. de l'inst. Pasteur*, xxi, p. 1005, 1907) the credit belongs to Duen-schmann (*Étude expérimentale sur le charbon symptomatique et ses relations avec l'oedème malin*, *Ann. de l'inst. Pasteur*, p. 482, 1895) who mentions the fact that Roux, under whose direction this work was carried out, had previously used serum (beef) to cultivate certain anaërobes. As a matter of fact Theobald Smith (*Das Gährungskölbchen in der Bakteriologie*, *Centralbl. f. Bakt.*, vii, p. 502, 1890) very distinctly mentions the fact that sterile animal tissue may be employed to advantage in the cultivation of certain obligate anaërobes and he actually employed fermentation tubes enriched in this manner at that time.

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the fermentation tube, he has added much to the value of this medium for bacterial research.<sup>1</sup>

Smith's published results have been obtained chiefly with pure cultures. Herter and Ward<sup>2</sup> have studied the gas volume with fermentation tubes inoculated with mixed intestinal flora and Herter<sup>3</sup> has studied the sediments derived from the inoculation of such tubes.

### TECHNIQUE.

(1) *Preparation of sample.* It is necessary at the start to have fresh specimens of stools for investigation—samples that have stood for some time or have been freely exposed to the air or to high temperatures are liable to change rapidly in their bacterial composition. Nonspore-forming anaërobes may rapidly succumb while, coincidentally, hardy forms multiply until they exist in abnormally large numbers, thus influencing very markedly the bacterial aspect of the result.

It is necessary in this particular line of investigation for several reasons to inoculate appropriate amounts of fecal material; the portion added must not be too great or the acids and other antagonistic products formed by the more rapidly growing facultative organisms will seriously inhibit the growth of the more strictly parasitic types; too small an amount, on the other hand, may fail to furnish a representative growth of significant microorganisms. The less numerous but perhaps extremely important forms may, without these precautions, be overlooked and missed entirely.

For routine purposes one gram of feces thoroughly emulsified in ten cubic centimeters of physiological saline solution is an

<sup>1</sup> Milk intended for the fermentation tube must be sterilized at least four successive times at appropriate intervals to insure the absence of resistant spore-forming bacteria which ordinarily escape observation. After sterilization and before inoculation milk fermentation tubes must be incubated several days at body temperature to test their sterility. See Smith, Brown and Walker: *The Fermentation Tube in the Study of Anaërobic Bacteria with Special Reference to Gas Production and the Use of Milk as a Culture Medium*, *Journ. Med. Research*, xiv, pp. 193-206, 1905.

<sup>2</sup> This *Journal*, i, p. 415, 1906.

<sup>3</sup> *The Common Bacterial Infections of the Digestive Tract*, 1907.

excellent dilution. Probably all types present that are capable of development in fecal media are thus represented.<sup>1</sup> If mucus is present in the stool it should be washed in sterile water and inoculated separately. One cubic centimeter of the suspension is placed in each tube.

Certain forms, as for example, the *B. bulgaricus* described by Metchnikoff, and many alkali-producing bacteria, will not grow in the ordinary fermentation media but usually develop rapidly in milk fermentation tubes.

The period of incubation is a very important factor. Experience has shown that during the first eighteen to twenty hours (rarely longer than this) the majority of the vegetative cells will be at their maximum growth; after this time, owing partly to antagonism, partly perhaps to the fact that the nutrient material that was carried over with the suspension is exhausted, many forms die out, while the more saprophytic organisms increase enormously.

The actual gas volume is rather less at twenty hours than at subsequent periods as a rule, but the relative value of this feature is at the maximum. The bacteria derived from ordinary stools, particularly of the colon type, tend to attain a more or less constant gas volume after forty-eight hours.

In plain bouillon without the addition of carbohydrates (particularly with media made from meat juice instead of meat extract) as a basis, gas is sometimes liberated after acidification of the culture with hydrochloric acid, although no gas was present during the incubation period. Also the addition of cystin to the medium tends to increase the gas volume. This gas is hydrogen sulphide, and its total amount may be estimated with a considerable degree of accuracy through the absorption effected by the addition of a soluble salt of a heavy metal.

The gas ratio is not an especially important characteristic in mixed fecal flora, much less so in fact than is the case with pure cultures. Frequently the volume is too small to measure, and

<sup>1</sup> The emulsion should be rapidly prepared and inoculated. Too long an exposure in the relatively aerobic saline solution may, and frequently does, eliminate many vegetative and nonspore-forming anaerobic forms, particularly those of infant flora, while the more vigorous aerobic bacteria increase rapidly.

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the inability to decide offhand which organisms are concerned in its elaboration makes the determination at best of doubtful value.

Of the greatest importance, on the contrary, is the character of the sediment. Smith<sup>1</sup> has shown that the deposit at the base of the closed arm is an excellent source of material for reinoculation with anaërobic bacteria (using pure cultures) and our observations have demonstrated in addition the fact that certain organisms assume characteristic appearances which in some instances are even diagnostic.

For making suitable smears the material is best removed by a finely drawn out capillary pipette, then spread upon slides, using the tip of the pipette as a spreader. The excess of solution runs back into the pipette by capillarity, leaving a thin, uniform smear which dries quickly and stains readily.

Gram's staining method followed by dilute carbol fuchsin or anilin-oil safranin furnishes the most distinctive preparations.

2. *Fermentative media.* For ordinary purposes, the regular dextrose, lactose and saccharose bouillons are employed. Freshly sterilized media are desirable because the anaerobic condition is much better developed in the closed arm in such media.

For special investigations, where it is necessary to be absolutely certain of the reaction of an organism upon different sugars, one of us (A. I. K.) has succeeded in eliminating the chief sources of error due to heating carbohydrate media, namely, the caramelization and inversion. This is conveniently accomplished by sterilizing the sugar separately (as a 10 per cent solution) by two passages through appropriate Berkefeld filters. The sugar solution is added to the nutrient bouillon (freed from fermentable substances by Smith's method) in such amounts that a 1 per cent solution of fermentable carbohydrate results.<sup>2</sup> The bouillon is

<sup>1</sup> Das Gährungskölbchen in der Bakteriologie, *Centralbl. f. Bakt.*, vii, pp. 502-506, 1890.

<sup>2</sup> Liborius (*Zeitschr. f. Hyg.*, i, p. 116, 165, 1886) was the first to recognize the importance of sugars and particularly dextrose, as a nutritive and reductive medium. He used 2 per cent, and his example has been followed without question by the majority of English, French and German investigators. Smith (*Centralbl. f. Bakt.*, xviii, p. 7, 1895; xxii, p. 49, 1897) also finds dextrose very important for the development of anaërobes but his

sterilized in fermentation tubes in the usual manner, adding rather less than usual to provide space for the carbohydrate which is introduced later.

Media made up in this way have one disadvantage, namely, the lack of absolute anaërobiosis that obtains when they are sterilized as one solution. This objection is more imaginary than real, however, because experience has shown that intestinal organisms capable of growing in pure culture in the ordinary single solution media will grow quite as well in the double solution medium.

#### RESULTS.

Herter and Ward<sup>1</sup> found, using dextrose, Schering's diabetin (dextrose-lævulose mixture), lactose and saccharose, the following average amounts of gas produced by normal stools (sixteen in all).

Dextrose.	Dextrose-lævulose.	Lactose.	Saccharose.
26.75	27.50	29.9	19.5 mm.

These fermentation tubes had anaerobic arms approximately 95 mm long. As a rule the lactose tube showed the most gas; saccharose the least. In conditions of disease the gas volume varied greatly—in certain instances 50 per cent of the vertical limb was filled; in other cases no gas was found.

Our own series indicate in addition that feces derived from many individuals harboring *Bact. Welchii* (the gas bacillus) may form extremely large volumes of gas—even 90 to 100 per cent of the whole tube—although the average is much less, usually about 45 mm. Pure cultures of *B. coli* form about 30 mm. of gas under similar conditions.

Coccal forms when present in numbers, generally inhibit gas formation: gas may even not be formed at all in certain cases, although gas-forming organisms are present. Normal infants, particularly those that are exclusively breast-fed, form as a rule rather less gas than adults. The volume varies with age and

results show that 2 per cent is too great a quantity and that 1 per cent is far preferable. Marino (*loc. cit.*) finds from 0.3 to 0.5 per cent even better and our experience indicates that the lesser amounts are preferable in many instances.

<sup>1</sup> *Loc. cit.*

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condition but fifteen to twenty millimeters represents fairly the average. The amount furthermore depends upon the relative proportion of *B. bifidus* present; if the latter organisms be abundant, less gas is formed, because this species produces sufficient acid to inhibit the growth of the ordinary gas-formers.

Diarrhœal stools vary in their gas production. In those cases where large numbers of cocci are brought down from higher levels of the intestine, relatively small gas volumes are the rule, while in similar movements, associated with large numbers of colon bacilli, or with organisms of the *lactis aërogenes* type, a much greater production takes place.

Mention has already been made of the fact that the fermentation tube is a particularly simple and efficient apparatus for cultivating anaërobic intestinal bacteria. Of these organisms a few will not grow in pure culture under the same conditions, although they usually thrive symbiotically with facultative anaërobes.

There can be no doubt that certain substances, particularly favorable for the growth of these more or less strictly parasitic forms are carried into the fermentation tube as a part of the fecal suspension, and during the first eighteen or twenty hours furnish a suitable pabulum for their growth—material, furthermore, which is not present in the fermentation tube as it is ordinarily made up. It is extremely probable that their growth is further aided by the presence of more readily growing bacteria which frequently render the tubes extremely anaërobic by the removal of the last traces of dissolved oxygen.

In the fermentation tube every transition from almost complete anaerobiosis to aërobiosis obtains and it is possible for bacteria to find almost any tension of oxygen from more or less complete saturation in the bulb to practically its entire absence in the closed arm.

With such favorable conditions—proper food supply and gaseous environment—the growths are very varied and in a measure representative of the organisms originally present in the feces. This fact is best appreciated after one examines the sediments, particularly those stained by Gram's method followed by the counter stain mentioned above.

The organisms are as a rule much more characteristic morphologically in the deposit at the bottom of the closed arm than is the

case in the feces from which they were derived, because the majority are in what may be termed the "active" vegetative stage. Bacteria in this condition are larger and more nearly typical than under conditions where they have become attenuated and degenerate in their morphology, as frequently happens in the case of constipated stools. The staining reactions also are much sharper and more distinctive at this period.

Perhaps the most striking example of the differentiation one may ordinarily meet with in a sediment from a fermentation tube is that shown by a common bacterium in infants' stools called by Tissier, its discoverer, *B. bifidus communis*. This organism is an anaërobic Gram-positive bacillus, frequently occurring with rather pointed ends in normal infants' stools; not readily recognized and not especially characteristic. Furthermore it is not an easy organism to cultivate in ordinary media. In fermentation tubes, however, it grows rapidly and at the end of eighteen hours shows the peculiarly striking bifid ends to which it owes its name. This fact, judging from the literature published upon the subject so far, has hitherto been unrecognized, but it appears to be characteristic, of great constancy, and a unique example of the value of such examinations. This organism will not grow, or at least only slightly, in fermentation media in pure culture. If, however, one adds a bit of sterile animal tissue, or inoculates directly from a stool, together with other bacteria, the growth is marked.

It is advisable, and frequently necessary, to use fermentation tubes containing plain bouillon instead of the regular fermentation media. Certain bacteria will not grow well where fermentable sugars are present, while others are rapidly eliminated as the medium becomes acid. *B. putrificus* is a good example of such an organism. Sediments derived from plain bouillon in fermentation tubes, particularly those which are rendered more suitable by the addition of sterile animal tissue, frequently show anaërobic growths that would not be included in carbohydrate solutions.

One point in connection with the fermentation tubes deserves special mention—gas volumes are frequently variable with the same individual and it is necessary to cover considerable periods of time before assigning a special value to this factor in individual cases or attaching much importance to deviations from the average.

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Among the organisms ordinarily met with in the feces, a few of the more important may be mentioned:

(1) *Bact. Welchii*, a thick, rather large, strongly Gram-positive bacillus. In large numbers they give rise to a considerable augmentation of the normal gas volume, so that the amount is frequently twice the normal.

An organism described by Herter<sup>1</sup> resembles the gas bacillus morphologically but does not form gas.

(2) *B. coli*. Short bacilli, about one micron in diameter, Gram-negative. These organisms usually determine the gas volume and it is chiefly to their action that the normal gas volume is due.

(3) *B. lactis aërogenes*, somewhat more oval than the colon bacillus and like that organism, Gram-negative. This form is not particularly common in the stools of adults<sup>2</sup> but is present usually in the excreta of bottle-fed infants and tends to increase the gas volume, if numerous.

(4) Coccal forms, usually Gram-positive. These bacteria produce as a rule considerable amounts of acid but no gas, and inhibit to a considerable degree the fermentative action of the above mentioned forms.

(5) A Gram-positive bacillus with bifid ends (*B. bifidus*). It is very common in the stools of breast-fed infants. When this organism is present in numbers, the amount of gas is usually considerably reduced. Its inhibitory action is due, as is the case with the coccal forms, to its excessive acid production.

In several instances, *B. bifidus* has been isolated from mucus, while the remainder of the stool was almost devoid of these forms.

The full significance of the fermentation-tube sediments cannot be regarded as completely worked out. It is a striking peculiarity of the growths in the sediments that they frequently do not show a multiplication of microorganisms closely representative of the varieties which are seen in the Gram-stained fields of the feces. This failure in correspondence between the characters of the dominant organisms in the fermentation tubes on the one

<sup>1</sup> *Loc. cit.*

<sup>2</sup> MacConkey (Lactose-fermenting Bacteria in Feces, *Journ. of Hygiene*, 1905, pp. 333-379) found it in 4 out of 625 lactose-fermenting cultures from normal stools, both animal and human.

hand and the feces themselves on the other, depends largely upon the fact that the nutrient conditions are ordinarily radically altered by the transfer to the fermentation media. This alteration in medium makes it possible for types of bacteria not obviously dominant in the feces, or, indeed, clearly in the minority, to gain a relatively prominent position under these conditions.

The fact that such a readjustment of types is liable to occur has important advantages and equally significant drawbacks. Without recognizing the disproportionate growth one might erroneously assume that a much larger portion of a certain flora is present than is the case; for example fecal fields may contain small numbers of *B. bifidus*, yet in the fermentation tubes they may be prominent. Again, the gas bacilli may undergo extensive multiplication in the fermentation tube despite the fact that the fecal material from which they were obtained contains them in moderate numbers only. Here again one sees the necessity for controlling the appearances obtained from the sediments of the fermentation tubes by means of cultures from the stools as well as by close examination of the Gram-stained fecal fields. Similar overgrowths occur with the coccal forms, *Mic. ovalis* (*enterocoque*), streptococci and staphylococci.

The disproportionate growth has its advantages as well as its disadvantages. Certain types which are significant although originally occurring in small numbers are thus brought to notice when otherwise they would be overlooked.

Experience has shown that overgrowths occurring during the first eighteen to twenty hours of incubation are due to the presence of significant numbers in the stools, capable of asserting themselves in the higher levels of the digestive tract, and capable of enormous proliferation under suitable nutrient conditions. An excellent example is again furnished by *Bact. Welchii*. In patients who have an infection of the intestinal tract with this organism there may be times of improvement when the numbers of this particular type in the feces is small, as shown by the microscopic examination of the fecal fields—so small, in fact, that if the observation were confined to the patient at this time, no suspicion would be excited of the existing tendency of overgrowth of these organisms in the intestine. Yet upon inoculation of the feces into fermentation tubes a prominent, active

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growth of these organisms is very liable to occur under these conditions.

In contrast with this is the following observation. The fecal fields from normal nurslings and bottle-fed children commonly show a few organisms having the morphology of the gas bacillus. That these bacteria belong in the class of the gas bacilli is made probable through the fact that by inoculating relatively large amounts of the feces into rabbit's ear-veins, with subsequent incubation (Welch-Nuttall test) the typical gas-liver will be developed. Inoculations into fermentation tubes made from feces of this type of case have, in our experience, failed uniformly to show overgrowths of this bacillus.

It should be clearly understood that the presence of moderate or even considerable numbers of *Bact. Welchii* does not necessarily lead to overgrowth in the fermentation tube.





## BACILLUS INFANTILIS (n. s.) AND ITS RELATION TO INFANTILISM.

(Plates VI and VII.)

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### *Introduction.*

The bacilli described in this paper, together with several variants were isolated from a series of chronic intestinal infections of obscure origin and etiology. The cases present a definite symptom-complex which has recently been described by Dr. C. A. Herter<sup>1</sup> and termed by him, "Infantilism from chronic intestinal infection." Clinically the pronounced symptoms are: an arrest of body development associated with only a slight retardation of cerebral development; marked abdominal distension (without, however, a noticeable enlargement of the spleen); slight to moderate secondary anæmia; marked bodily and mental fatigue, brought on by relatively slight exertion, and disturbances of intestinal function manifested usually by repeated diarrhoeal attacks and impaired powers of absorption.

Chemically, the salient features are: the presence of excessive amounts of indican and phenolic bodies in the urine; indolacetic acid occurs in some cases and may be present in greatly excessive amounts. The aromatic oxyacids may also occur in excessive quantities.

In patients receiving considerable fat in the diet, the feces contain a large excess of neutral fats, fatty acids and soaps, the excretion of the latter leading to considerable losses of calcium and magnesium to the body. This loss of calcium and magne-

<sup>1</sup> Herter: *Infantilism*, The Macmillan Company, 1908.

sium furnishes a logical explanation of the retardation of skeletal development.

*Part I: Microscopical Examination of Infantilism Stools*

Representative fields of stools from typical cases of infantilism, stained by the Gram-Weigert method (Gram-stain followed by dilute carbol fuchsin, as a counter-stain) are strongly Gram-positive, and recall similar pictures described by Escherich<sup>1</sup> in his cases of *Blaue Bacillöse* with respect to the great diminution or even absence of Gram-negative cocco-bacilli of the coli-aërogenes type, and the dominantly Gram-positive character of the fields. Certain differences are detectable, however, upon closer scrutiny. The Gram-positive curved bacilli which are characteristic of *Blaue Bacillöse* cases are few in number or even absent in infantilism stools, while the most distinctive organisms in the latter are straight rods measuring from 0.50 to 0.75 microns in diameter, and from 2.5 to 3.75 microns in length. These bacteria are Gram-positive and occur singly or in pairs, rarely in short chains of from four to six elements. They present no distinctive peculiarities of arrangement, although they are frequently collected into rather sharply defined masses.

It is not uncommon to find signs of degeneration among these bacilli, particularly if they are derived from constipated stools.<sup>2</sup> In such instances the bacilli are more or less irregular in outline and do not stain uniformly, some portions of the cytoplasm remaining Gram-positive, while other portions stain faintly with the counter-stain or not at all.

Cultural methods demonstrate these bacilli to be *B. infantilis*, *B. bifidus*<sup>3</sup> or *B. acidophilus*.<sup>4</sup> One type may predominate or all may be represented at the same time.

<sup>1</sup> Escherich: *Jahrb. f. Kinderheilk.*, lii, pp. 1, et seq., 1900.

<sup>2</sup> Herter and Kendall: *This Journal*, v, p. 289, 1908.

<sup>3</sup> Tissier: *La flore intestinale du nourrisson*, Paris, 1900. Tissier gave this organism the name *B. bifidus communis*, a trinomial. This name, being a trinomial, violates the law of botanical nomenclature and is incorrect. Inasmuch as the term *bifidus* expresses tersely the most prominent characteristic of the bacillus, it should be retained as the specific name, eliminating the superfluous term, *communis*.

<sup>4</sup> Moro: *Jahrb. f. Kinderheilk.*, lii, p. 38, 1900; *Wien. klin. Wochenschr.*, no. 5, 1900.

It should be possible, theoretically, to make a morphological diagnosis between *B. bifidus* and *B. acidophilus* on the one hand, and *B. infantilis* on the other. Tissier claims that *B. bifidus* occurs typically with pointed ends and not infrequently one sees pairs of bacilli lying at an angle with each other—the so-called geniculate arrangement. *B. acidophilus* resembles *B. bifidus* somewhat morphologically, but the bacilli occur in sharply circumscribed masses, instead of geniculate pairs in stools. *B. infantilis*, on the contrary, has definitely rounded ends; clostridial forms are uncommon, and the bacilli do not occur in regular alignment as a rule.

The various manipulations to which stools are subjected prior to microscopical examination are usually sufficient to disturb the original orientation of the bacilli, while their morphology varies somewhat with the consistency of the stool, so that the final criteria of their differentiation and recognition must be based upon cultural methods.

Branched forms are not uncommon in the stools of infantile patients, and rarely one sees a peculiar modification of the branched form to which the term "antennate" has been applied. The branched forms may be referable to either *B. bifidus* or *B. infantilis*; each organism produces under certain undetermined conditions branched forms in stools. It is not impossible that some development has taken place after the stool was passed in such instances, because many of the bacteria show signs of degenerative changes which are known to occur when they are exposed to unfavorable conditions, outside of the body.

The "antennate" form is unusual in the feces and so far as the writer is aware, no form similar in appearance has been described for any known bacillus. The cell body is slightly spindle-shaped (clostridial) in outline and possesses a deeply staining metachromatic granule at one or both ends of the rod. The cytoplasm between these granules takes the stain feebly or even not at all, and suggests both by its position and general appearance a presporogenic body. From one, rarely both, of the metachromatic granules processes arise, which may be one, two or three in number; two is the most usual arrangement. These outgrowths are slightly curved, slender and elongated; they do not project in the same general direction as that of the long axis of the cell proper, but rather at an obtuse angle, resembling in a striking manner the antennæ of certain beetles. This resemblance is further accentuated by the irregular manner in which they take the stain, which gives them a jointed or articu-

lated appearance. The same forms have been encountered in old broth cultures of this organism, and they are probably to be regarded as involution forms. If this supposition be correct the antennate forms have no particular significance in the life history of the bacterium.

Besides *B. infantilis*, *B. bifidus* and *B. acidophilus* one notices frequently in the feces of infantilism patients large, Gram-positive cocci which occur typically in pairs, less commonly in short chains. They measure about one micron in diameter and their ends are more or less pointed. Their elongated appearance has led the French writers to refer to them as "*Flamme de Bougie*"—an epithet which is very expressive of their peculiar morphology. These organisms are known as the "*Enterocoque*" of Thiercelin,<sup>1</sup> or the "*Micrococcus ovalis*" of Escherich;<sup>2</sup> and, according to Kruse<sup>3</sup> are identical with the *Streptococcus* of Hirsch-Libmann. Kruse proposed the name, "*Streptococcus lacticus*."

These organisms are the Gram-positive types most frequently found in the dejecta of infantilism cases, and *B. bifidus*, *B. acidophilus* and *Micrococcus ovalis* represent the acidophilic bacteria which are characteristic of well marked clinical states of this kind. It is noteworthy, as Herter<sup>4</sup> has pointed out, that the latter organisms are the dominant ones present in the stools of breast-fed infants—a fact that is of great importance in this connection because the bodily development and intestinal flora seem to be related to each other.

### *Part II: Methods for Isolating Bacillus Infantilis.*

The first culture of *Bacillus infantilis* was obtained from a stool in which they were present in unusual numbers, and no difficulty was experienced in obtaining fresh strains for nearly a month. At the end of that time, however, the bacterial flora changed and it became increasingly difficult to obtain cultures by the usual method of fishing plates.

<sup>1</sup> *Compt. rend. de la soc. de biol.*, April 15 and June 24, 1889.

<sup>2</sup> *Darmbakterien des Säuglings*, Stuttgart, p. 89, 1886.

<sup>3</sup> *Centralbl. f. Bact.*, xxxiv, p. 737, 1903.

<sup>4</sup> *Loc. cit.*

The colonies produced by *B. infantilis* are reasonably distinctive when they occur in pure culture, or when there are only a few other types of organisms in the same plate. As the number of colonies, either of the same type of organisms, or different organisms, increases, they lose their characteristic appearance, and it becomes increasingly difficult to make even a probable diagnosis by simple observation alone.

Again, contrary to generally accepted ideas, it has been found that not only do colonies of the same organisms vary in appearance in the same plate, but also that radically different types of bacteria produce colonies which are indistinguishable.

These differences are explainable upon two hypotheses. In the first place the rigidity of the medium has an extremely important rôle. In low percentage agar, those bacteria which are strongly motile, together with the forms that produce chains readily, will grow in colonies that are characterized by considerable lateral expansion. This is particularly the case in surface colonies. Submerged growths to a lesser extent obey the same general laws. On the other hand, particularly among the intestinal bacteria, the metabolic products of one type of organism may react by diffusion upon adjacent bacteria, resulting in a mutual inhibition of growth. The strongly acidogenic bacteria, for example, particularly when grown in media which contain fermentable sugars, will seriously interfere with the growth of alkali-producing types.

A consideration of the facts presented in the preceding pages indicates clearly the necessity of examining relatively large numbers of colonies, particularly in the study of unusual cases of intestinal disease. Obviously this is impracticable in actual work, where several cases are under observation at the same time. What is needed is a method whereby one may study the morphology of the bacteria from a representative majority of all the colonies which have developed in the various media, and to be able to return to specified colonies which through this preliminary examination have been demonstrated to consist of unusual types of bacteria.

This may be accomplished in the following manner. Plates showing from 20 to 50 well-separated colonies are marked with a wax pencil in such a way that the projections of these colonies upon the bottom of the plate are enclosed in a small ring. This procedure is kept up until a sufficient number have been selected

for examination. The colonies are then numbered in sequence, beginning with 1 and continuing until all are numbered, remembering to place a line under the figure 6 to distinguish it from the 9.

An ordinary microscopical slide is now ruled into squares, the size and number depending upon the number of colonies which it is proposed to study. A small amount of growth from each colony is emulsified in a drop of sterile water in its appropriate square, the films are dried, stained by the Gram-Weigert method and examined microscopically. This examination will indicate the general type of organism in each instance, and not infrequently demonstrate the fact that certain colonies are composed of two distinct kinds of bacteria. Having thus not only recognized the morphological peculiarities of the organisms, but also having a fair idea of the relative purity of each colony, one may select those organisms which it is desirable to study in detail, resorting to the colonies from which they were derived and subculturing them at convenience. This procedure has been very helpful and it is extremely probable that the rather large number of apparently undescribed forms which have been met with in this work, have been successfully isolated because attention has been paid to the study of large numbers of colonies which upon superficial examination show no macroscopical differences. By adopting this procedure, one may be reasonably certain that at least the dominant forms which will grow in artificial media, will be detected. In order to make a systematic investigation, however, it is absolutely necessary to use media containing either dextrose (lactose is usually better for infants' stools) or other easily fermentable carbohydrate for the fermenting and acid producing organisms, and carbohydrate-free media for the alkali producers. One may thus eliminate to a considerable extent the effects of antagonism between the acidogens and the alkali producers.

### *Part III: The Biology of Bacillus Infantilis.*

This organism is a motile, aerobic, facultative anaerobic, spore-forming, Gram-positive, non-capsulated bacillus. It produces acid in dextrose and saccharose, but no gas.

*Morphology.* The bacillus measures from 0.50 to 0.75 micron in diameter, and from 2.5 to 3.5 microns in length.

Upon solid media the bacilli appear as rather short, plump rods with rounded ends. They occur singly or in pairs, rarely in short chains. Spores are readily formed in such media, in the presence of free oxygen. In fluid media, on the other hand, spores are not formed as a rule, and in anaërobic cultures spore-formation has never been observed. This need for oxygen probably explains the inability of *B. infantilis* to form spores in the intestinal tract. Repeated attempts to obtain cultures of this organism after destruction of the vegetative cells by heating suspensions of the stool to 80° C. for 10 minutes have failed except in two instances. It should be stated that it was possible to isolate these organisms in unheated suspensions made from the same stool. The two cases in which spores were present do not necessarily invalidate this view. The stools were 24 hours old at the time they were examined and it is perfectly evident that the necessary conditions for spore formation were present.

The spores are central, oval, and cause a slight bulging of the cell; this enlargement is not great enough, however, to give the organisms the appearance of clostridia.

The bacteria found in the condensation water of agar, as well as in fluid media in general, differ conspicuously in appearance and staining<sup>1</sup> reaction from those grown upon solid media. In the latter instance the bacilli are relatively short and thick; in

<sup>1</sup> At first the difference in staining reaction between organisms derived from the slanted surface of agar, on the one hand, and the condensation water of the same culture, on the other, led to the suspicion that a contamination had occurred. Experiments definitely reproducing the phenomena were made, however, and it was discovered that this same property is common to a number of Gram-positive bacteria of intestinal origin. The differences observable are very striking in some instances. When the bacteria are kept upon artificial media for several successive transfers the variations tend to disappear and the organisms assume a uniform appearance whether they are obtained from the slanted surface or from the condensation water. It is probable that the sudden change from the intestinal environment to that of ordinary media is responsible for the lack of stability, and as the bacteria are in a weakened state, changes in their environment as slight as those existing in slanted surface and condensation water will produce noticeable responses in bacterial growth.

the former they are longer, thinner, and conspicuously Gram-negative. The staining reaction has been described previously in this paper, and it will suffice to say that the "punctate" appearance which one sees in stools can be reproduced exactly in fluid media.<sup>1</sup>

*Temperature relations.* *B. infantilis* grows most characteristically and luxuriantly at the body temperature; at lower temperatures the growth is scanty, and at 18° C. it practically ceases.

*Anaërobiosis.* The organism is an aërobe, although it can develop in hydrogen, carbon dioxide and in the depth of anaërobic stab cultures. Certain peculiarities concerning its oxygen needs will be described later on, in the section upon fermentation.

*Agar slant.* Upon dextrose agar (slanted) there is produced a spreading, gray, smooth, opaque, shining layer. In the condensation water growth is abundant and usually associated with the production of a pellicle. The medium becomes brown in older cultures and exhibits a tendency to the production of a well marked opacity. These two phenomena are distinct and apparently have no relation to each other.

Three variants have been noticed. The first is characterized by the production of a viscosity due to the ability of the bacteria to form adhesive, easily drawn out threads. The second variant grows poorly upon the slanted surface, and appears as a delicate, translucent, shining filiform layer which never produces the browning of the medium referred to above. Organisms of this type are chemically and morphologically identical with the luxuriantly growing varieties. They may be regarded as strains which have not become thoroughly habituated to artificial media. It should be stated that the latter type grows better in hydrogen than in aërobic conditions. The third variant is a chromogen. The pigment, which is soluble in the medium, and occurs in the upper layers, where oxygen is present, is red-brown, recalling the pigment produced by an organism erroneously named *B. lactis erythrogenes*.

The chromogenic variant may arise spontaneously. In fact

<sup>1</sup> Prolonged cultivation in artificial media causes the organism to lose its ability to take the Gram-stain; in this respect it agrees with several well known bacteria of similar origin.

a chromogenic strain has never been isolated from infantilis stools. Culturally, morphologically and chemically these strains are identical with typical infantilis cultures.<sup>1</sup>

*Gelatin stab.* Growth in gelatin stabs is scanty. In dextrose gelatin it is more vigorous. At temperatures ranging from 18° to 22° C. development takes place very slowly, and there is no appreciable liquefaction. As the temperature rises, however, the organisms develop more rapidly, and at 24° liquefaction may take place at the end of six days. The liquefaction is very slow and frequently is manifested merely by the presence of a slight infundibuliform, dry depression. Evaporation usually progresses parallel to peptonization, so that no fluid remains in the cavity. Rarely a small amount of liquid remains. In cultures which have been kept at room temperature for several months liquefaction may progress to such an extent that a funnel-shaped depression measuring half a centimeter in depth may result. The medium is not favorable for the development of the bacillus, however, and certain strains do not liquefy at all, but produce merely a softening of the medium. The reaction becomes alkaline.

*Milk.* Freshly isolated strains produce a transient acidity followed by a return to the neutral point. In older cultures the reaction becomes alkaline. The primary acidity is doubtless due to the fermentation of carbohydrates (hexoses) present in milk, due to the hydrolysis of lactose. Prolonged cultivation of the bacteria in milk is associated with more marked changes in the medium. A gelatinous coagulum is formed which is not marked and which usually requires a boiling temperature to demonstrate. This stage is followed by a gradual solution of the coagulum, an increased alkalinity and a gradual thinning of the whole medium. The thin, alkaline fluid resembles that produced by some varieties of the paratyphoid bacillus.

<sup>1</sup> Beijerinck (*Kon. Akad. von Wetenschappen te Amsterdam*, Oct. 27, 1900, and Rodet (*De la variabilité dans les microbes*, Paris, 1894) have studied the question of bacterial variation, and define three types, Degeneration, Transformation and Variation, in the narrow sense. The production *de novo* of pigment in *Bacillus infantilis* seems to correspond with the latter type of variation, which is defined as "the assumption of a new characteristic (by a single individual of a single strain) which remains constant. Insufficient or improper media, or the excess of excretory products in old cultures may be the cause of this phenomenon."

Milk is not, however, a favorable medium in which to cultivate *Bacillus infantilis* and the changes produced by this organism in milk are not distinctive.

*Potato.* The growth is luxuriant, raised, shining, smooth, brownish and is attended with a darkening of the potato. The reaction is not appreciably changed.

*Fermentation media.* Dextrose and saccharose are fermented with the production of acid, but no gas. Lactose is only slightly attacked. The acidity reaches its maximum about the fourth day, and after that time there is a gradual return to the neutral point, or even in extreme cases to alkalinity. The explanation is probably to be correlated with the ability of this organism to produce ammonia and primary amines. During the first few days there is a marked growth in the closed arm (in dextrose and saccharose fermentation tubes) and it is during this period that the acidity rapidly increases. At the end of the time specified the bacteria grow more abundantly in the open arm of the tube and this aerobic growth is associated with a large ammonia production.

Lactose fermentation media are not favorable to anaerobic growth of *B. infantilis* and it is a noteworthy fact that in the closed arm growth is practically wanting. There is, however, evidence that the organisms are proliferating.<sup>1</sup> A pellicle makes its appearance within 30 hours after inoculation, which suggests that the organism cannot derive its oxygen through the combustion of lactose, although it can thrive in an atmosphere devoid of oxygen if either dextrose or saccharose is present. Confirmation of this view is indicated by the formation of a similar pellicle in plain broth. Here again there is no substance from which the organism can derive its oxygen. Hence it is forced to obtain it from the air. The wrinkling of the pellicle and the almost total absence of turbidity (the organism is actively motile) point strongly to the correctness of this explanation.

*Plate cultures.* Although *B. infantilis* is an aerobic organism,

<sup>1</sup> This inability to develop in lactose is significant when one considers that this sugar rather than dextrose or saccharose is the important carbohydrate in the dietary of young children. The normal intestinal bacteria on the other hand are able to grow luxuriantly in media containing lactose.



FIG. 1



FIG. 2



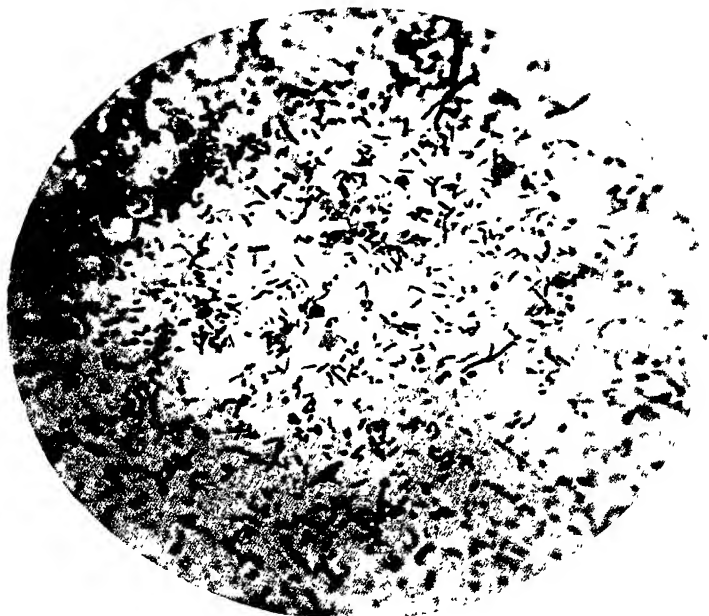


FIG. 3

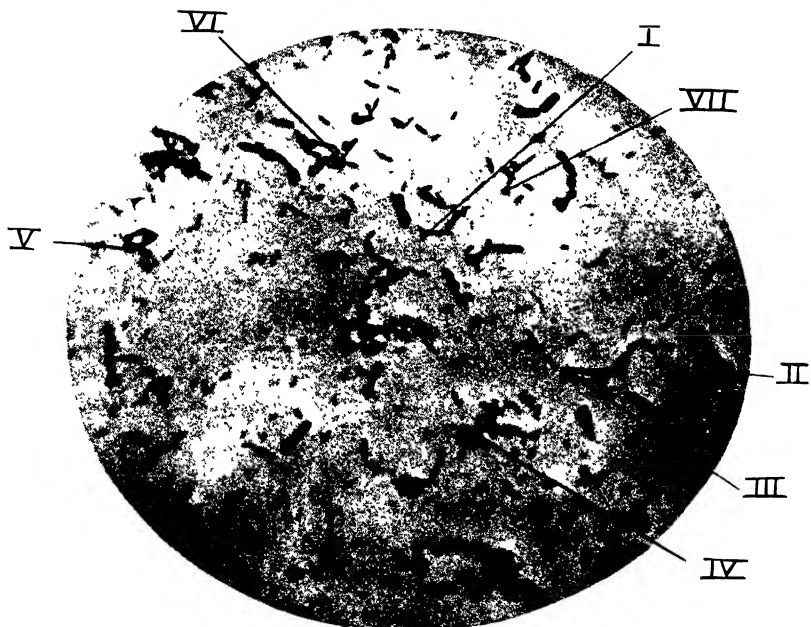


FIG. 4



it usually grows beneath the surface in dextrose agar plates. (Plain agar plates, on the contrary, show relatively more surface colonies.) The submerged colonies are lenticular, oval or even round. They are opaque, yellowish or gray (depending upon the depth below the surface) with delicately ciliate edges. The colonies are surrounded by an opaque area which resembles a halo. Microscopically the edges are seen to be filamentous and the general look of the colony suggests an aggregation of filaments, presenting a floccose appearance. Klatsch preparations, however, do not show the presence of long filaments; the individual bacteria, on the contrary, have a disjointed appearance and even short chains are uncommon. Surface colonies are translucent to opaque, and round, with irregular edges. Their appearance depends largely upon the relative density of the medium and particularly upon the amount of moisture present. If the latter is excessive, the colonies spread and resemble delicate films. The colonies are not characteristic, particularly when there are several different kinds of bacteria present in the same plate, and it is only by examining individual colonies microscopically that one can safely make the diagnosis.

*Biochemistry.* The biochemistry will be discussed in detail in a subsequent paper. A summary of the principal products, however, is here appended for completeness.

*Bacillus infantilis* is a powerful alkali-producer. Ammonia and an unidentified primary amine are the chief basic bodies found in cultures. The organism does not produce indol, skatol, phenolic bodies, mercaptan, hydrogen sulphide, alcohol, acetone or aldehyde. It produces lactic and succinic acids from dextrose, together with small amounts of volatile acids of unknown compositions. It gives the Voges-Proskauer reaction<sup>1</sup> in peptone media.

Certain strains of *Bacillus infantilis* are, at the time of their isolation, strongly acidophilic, or, better, have acquired the ability to withstand unusual amounts of acid. A sharp distinction should be drawn between bacteria which grow well in moderate amounts of acid, e. g., *B. acidophilus*, and those organisms which—originally not acidophilic—become by changes

<sup>1</sup> Harden and Walpole (*Proc. Roy. Soc.*, lxxxvii, p. 424, 1906) have shown that this reaction is due to the presence of methylacetylcarbinol.

in their environment able to withstand and to develop in great amounts of acid. Several cultures were obtained from a case of infantilism in association with *Bacillus acidophilus* and *Micrococcus ovalis*. All of these organisms were able to develop in a broth medium containing acetic acid of such a strength that three cubic centimeters of normal sodium hydroxide were required to neutralize one hundred cubic centimeters of the medium. The organisms were obtained in the following manner. A small amount of feces was emulsified in a tube of the acetic acid broth, and incubated two days. A loopful of the first broth culture was introduced into a second tube; after two days a third culture was prepared from the second. At the end of the last period of two days, the organisms were plated out and cultures were obtained in the usual manner. There can be no doubt that there was a decided development of *B. infantilis* during the progress of the experiment and considerable numbers of colonies were obtained on the plates. Subsequent cultural and chemical studies showed that they were identical with the first strains of this organism which had been isolated in the usual manner, in neutral media. The striking fact is that two transfers in ordinary media so changed the acid-resisting ability of the "acid infantilis" that it would not develop in media having an acidity greater than that corresponding to 1.25 cc. normal acid per 100 cc.<sup>1</sup>

<sup>1</sup> This experiment has a distinct value in relation to the question of bacterial conditions obtaining in the intestinal tract. In no other pathological conditions are the bacterial flora so complex and dependent in their ensemble upon obscure changes in environment. These environmental changes may consist of altered intestinal secretions, changes in diet or of bacterial antagonism and symbiosis or of combinations of these factors. Apparently slight nutritional alterations are frequently accompanied by surprising modifications in the bacterial behavior, the bacterial response seeming to be out of proportion to the intensity of the stimuli.

Although we are ignorant of the fundamental principles which dominate the bacterial flora of the intestinal tract, evidence is slowly accumulating which points to a symbiotic relationship between the host on the one hand, and the dominant types of bacteria on the other, at least in normal, healthy individuals. Undoubtedly diet plays a prominent part in determining which bacteria shall increase and which shall be inhibited.

When one stops to consider the extraordinary number of bacteria, dead and living, that are voided every day in the feces, and compares

The discovery of the acidophoric strain of *B. infantilis* brought up the question of antagonistic and symbiotic relations between this organism and bacteria with which it is commonly associated in the intestinal tract. Naturally it is impossible to reproduce conditions comparable to those existing in the intestines, but attempts have been made to compare the behavior of pure cultures of a few representative bacteria with combinations of the same bacteria grown together.

*Bacillus coli* and *Micrococcus ovalis* (enterococcus) have been chosen as the types to compare with *B. infantilis*, and all observations have been made in fermentation tubes, where every transition can be obtained from almost absolute anaerobiosis to complete saturation with free oxygen.

this figure with the relatively few organisms which are ingested during the same period, some idea of the enormous proliferation which takes place in the intestinal tract will be obtained

Escherich, Tissier and Moro have studied the kinds of bacteria present in the dejecta of normal nurslings, and find that certain well defined types of organisms are regularly present to the more or less complete exclusion of other types. Tissier has noticed that the vast majority of these bacteria are acidophilic, and he believes that the high degree of acidity which they can resist while still thriving acts as a deterrent to the growth of foreign organisms. When an infant suffers from an intestinal upset, the normal bacterial conditions are disturbed, and new varieties make their appearance. As conditions return to the normal, there is a gradual corresponding reappearance of the customary flora, associated with the disappearance of the abnormal types.

If, however, the abnormal conditions persist, the bacteria associated with the change may become habituated in the intestine, and eventually may replace, in part at least, the normal inhabitants. This invasion, as the writer has noticed in a number of instances, is accompanied by reciprocal modifications in both the normal and the invading organisms. This change is manifested by the ability of the two types of organisms to grow in the presence of each other, even if originally this was impossible. One may demonstrate this fact by comparing corresponding strains from normal intestines with the "modified" varieties. Usually one finds that the "normal" organisms will not thrive in such cultures.

This fact points to deep seated modifications in the biology of both the invading bacteria and the normal obligate organisms and undoubtedly these mutual adaptations explain in part the persistence of unusual types of bacteria in chronic intestinal diseases.

The facts brought forward indicate the necessity of studying with great thoroughness those cases of intestinal derangement accompanied by evidences of abnormal bacterial development.

Protocols of two experiments are reproduced. The experiments were carried on during four days, both in dextrose and lactose. All of the bacteria used in these experiments were given preliminary cultivation in dextrose broth to insure a high degree of reproductive growth.

These experiments emphasize once again the inability of *B. infantilis* to ferment lactose. This is indicated not only by the absence of turbidity in the closed arm of the fermentation tube, but by the production of a thick pellicle upon the free surface of the medium in the bulb. There is a corresponding absence of inhibitory action of this organism upon *B. coli* and *Micrococcus ovalis*, although in dextrose under similar conditions the restraining activity is marked.

It is obvious, then, that the marked decrease in gas formation which is observed in cases of infantilism is due to some factor other than the mere presence of *B. infantilis* because this reduction is as marked in lactose as in dextrose. It is extremely probable that *B. bifidus* is instrumental in preventing the development of bacilli of the colon-aërogenes types, and inasmuch as the latter organisms are the dominant gas formers of the intestinal tracts of young children, it is logical to associate the comparative absence of the gas-formers and the simultaneous presence of *B. bifidus* with the non-appearance of gas by the fecal bacteria from well marked infantilism cases.

Unfortunately the rôle of *B. bifidus* in this connection must, for the present at least, rest upon purely circumstantial evidence. It is not possible with our present methods to prepare media in which *B. bifidus* and *B. coli* (or other facultative organisms) shall under the same conditions grow with the same relative intensity. Obviously, if one organism develops more rapidly than the others it is out of the question to draw correct conclusions with reference to their antagonistic properties.

*The systematic position of B. infantilis.* *B. infantilis* differs in essential characteristics from any previously described organism known to the writer. Particular attention has been paid to the previously published descriptions of bacteria found in nurslings' stools, both in health and disease, and the works of Tissier,<sup>1</sup>

<sup>1</sup> *Loc. cit.*, p. 263, 1900.

## EXPERIMENT I.

Culture.	Day.	DEXTROSE.				LACTOSE.			
		Gas in mm.	Turb. clsd. arm.	React.	Pell.	Gas in mm.	Turb. clsd. arm.	React.	Pell.
Infantilis.....	1	—	+	acid	—	—	—	±	++
	2	—	+	"	—	—	—	±	++
	3	—	+	"	+	—	—	alk.	++
	4	—	+	+ 1.2	+	—	—	—0.5	++
Coli.....	1	16	+	acid	—	15	+	acid	—
	2	18	+	"	—	20	+	"	—
	3	22	+	"	—	24	+	"	—
	4	22	+	+ 2.25	—	25	+	+ 1.5	—
Ovalis.....	1	—	+	acid	—	—	+	acid	—
	2	—	+	"	—	—	+	"	—
	3	—	+	"	—	—	+	"	—
	4	—	+	+ 1.15	—	—	+	+ 1.1	—
Infantilis and Coli...	1	12*	+	acid	—	11	+	acid	—
	2	12	+	"	—	27	+	"	—
	3	12	+	"	—	32	+	"	—
	4	12	+	+ 2.2	—	32	+	+ 1.6	—
Infantilis and Ovalis	1	—	+	acid	—	—	+	acid	—
	2	—	+	"	—	—	+	"	—
	3	—	+	"	—	—	+	"	—
	4	—	+	+ 2.5	—	—	+	+ 3.0	—
Coli and Ovalis.....	1	22	+	acid	—	16	+	acid	—
	2	23	+	"	—	26	+	"	—
	3	24	+	"	—	27	+	"	—
	4	20	+	+ 3.4	—	25	+	+ 3.1	—
Infantilis, Coli and Ovalis.....	1	12	+	acid	—	19	+	acid	—
	2	11	+	"	—	21	+	"	—
	3	11	+	"	—	21	+	"	—
	4	10	+	+ 3.5	—	21	+	+ 3.3	—

\* Trials with a freshly isolated culture of *B. infantilis* showed a much greater inhibition of gas production. The total volume of gas produced in the preliminary experiments in those tubes containing *B. coli* and *B. infantilis* only showed 5 mm. of gas, although *B. coli* alone produced 26 mm. in the same media.

## Bacillus Infantilis

## EXPERIMENT II.

Culture.	Day.	DEXTROSE.				LACTOSE.			
		Gas in mm.	Turb. cld. arm.	React.	Pell.	Gas in mm.	Turb. cld. arm.	React.	Pell.
Infantilis.....	1	—	+	acid	—	—	—	±	++
	2	—	+	"	—	—	—	alk.	++
	3	—	+	"	+	—	—	"	++
	4	—	+	+1.3	+	—	±	-0.6	++
Coli.....	1	26	+	acid	—	35	+	acid	—
	2	29	+	"	—	37	+	"	—
	3	28	+	"	—	36	+	"	—
	4	28	+	+2.45	—	34	+	+3.0	—
Ovalis....	1	—	+	acid	—	—	+	acid	—
	2	—	+	"	—	—	+	"	—
	3	—	+	"	—	—	+	"	—
	4	—	+	+1.05	—	—	+	+1.50	—
Infantilis and Coli..	1	21	+	acid	—	29	+	acid	—
	2	23	+	"	—	33	+	"	—
	3	22	+	"	—	31	+	"	—
	4	22	+	+2.20	—	31	+	+2.65	—
Infantilis and Ovalis	1	—	+	acid	—	—	+	acid	—
	2	—	+	"	—	—	+	"	—
	3	—	+	"	—	—	+	"	—
	4	—	+	+2.7	—	—	+	+2.8	—
Coli and Ovalis.....	1	25	+	acid	—	28	+	acid	—
	2	26	+	"	—	32	+	"	—
	3	22	+	"	—	30	+	"	—
	4	22	+	+3.8	—	30	+	+3.0	—
Infantilis, Coli Ovalis .....	1	11	+	acid	—	30	+	acid	—
	2	11	+	"	—	31	+	"	—
	3	9	+	"	—	28	+	"	—
	4	10	+	+3.45	—	28	+	+3.9	—

Escherich,<sup>1</sup> Finkelstein,<sup>2</sup> Moro<sup>3</sup> and Salge,<sup>4</sup> have been freely consulted.

An organism described by Salge attracted especial attention. He found it in cases of catarrh of the small intestine and discovered that it had the property of breaking up sodium oleate into lower fatty acids, and that the presence of fats increased its fermentative powers. Inasmuch as an abnormal excretion of fatty acids and fats is a feature of infantilism, a possible relationship between infantilis and Salge's bacillus was suspected. Subsequent investigation, however, showed that there is very little resemblance between the two organisms.

Finkelstein described an acidophilic organism which he obtained from cases of *Blaue Bacilliose*, but it is evident that his bacillus is closely related to *B. acidophilus*. (This organism, as already pointed out, occurs in infantilism stools together with *B. infantilis*.)

*B. infantilis* belongs to the *B. subtilis* group. It produces resistant spores, forms alkali in milk and non-saccharine media, and does not produce gas. It is, however, smaller than any hitherto described subtiloid bacillus, and its ability to liquefy gelatin is much less marked than is the case with other members of this group. It should be mentioned in this connection that an organism having the morphological and cultural characters of *B. infantilis* has been isolated by the writer from canned plums. It differs from *B. infantilis*, however, by its relatively rapid peptonizing action in gelatin and its inability to form primary amines. This organism seems to be a connecting link which not only indicates the relation of *B. infantilis* to *B. subtilis* group but emphasizes the lines along which the latter organism tends to deviate from the typical organisms of the group.

#### *Part IV: The Relation of B. Infantilis to Infantilism.*

*B. infantilis* is a spore-forming organism, and as Theobald Smith<sup>5</sup> has pointed out, spore-forming bacteria are not, as a rule,

<sup>1</sup> *Loc. cit.*

<sup>2</sup> *Deutsch. med. Wochenschr.*, p. 263, 1900.

<sup>3</sup> *Loc. cit.*

<sup>4</sup> *Jahrb. f. Kinderheilk.*, lix, p. 399, 1904.

<sup>5</sup> Theobald Smith: Some Problems in the Life History of Pathogenic Microorganisms, *Amer. Med.*, viii, pp. 711-718, 1904.

obligate parasites, at least in man. The organism has not only been found in the stools of all the typical cases of infantilism so far examined<sup>1</sup> but also in the dejecta of normal nurslings derived from various sources, although in the latter instances the bacilli were very few in number and were not obtained from all the feces examined.

The fact that these cases, both pathological and normal, represent a fairly wide geographical area seems to indicate that *B. infantilis* may be well distributed in nature. In some instances it apparently finds a favorable environment in the intestinal tract of young children, obtains a foothold and proliferates there. In spite of this proliferation, however, specific agglutinins are apparently not produced, and although it certainly occurs in large numbers at certain periods of the disease, evidence is strongly in favor of the view that it is non-invasive.

One is not justified, however, upon these grounds in concluding that there is no etiological relationship. Feeding experiments upon a dog resulted in the establishment of a well marked diarrhoea<sup>2</sup> associated with the appearance of *B. infantilis* in the stools. A monkey, whose diet was carefully regulated, reacted even more strikingly. The movements became soft, pale in color, strongly Gram-positive, and there was simultaneously a marked increase of acidogenic bacteria (first of the *Micrococcus ovalis* type, then a rapid rise in the proportion of *B. acidophilus*, associated with a moderate number of *B. bifidus*). There was a corresponding diminution in the Gram-negative coli-aërogenes type of bacilli. That is, there was bacterially a decided tendency toward the development of the infantilism type of stool.<sup>3</sup> These effects are

<sup>1</sup> For list of cases, see Herter, *loc. cit.*

<sup>2</sup> It is interesting to note in this connection that Ardoïn (Thèse de Paris, p. 78, 1898) and Spielgelberg (*Jahrb. f. Kinderheilk.*, xlix, p. 194, 1895) have isolated and described bacteria belonging to the subtilis group which cause decided diarrhoeal disturbances, particularly in young children. Certain cases quoted by these investigators were characterized by the relative abundance of subtiloid organisms in the stools.

<sup>3</sup> The appearance of acidophilic bacteria following so closely upon that of *B. infantilis* is perhaps the most noteworthy feature of these feeding experiments, because cases of infantilism usually run the same course, bacterially. The explanation of this bacterial sequence is not known and the data available at the present time do not justify more than the bare statement of the fact.

transient and tend to disappear after a few days, but they may be reproduced by fresh infection with *B. infantilis*. These diarrhoeal disturbances are conceivably due to the irritant action of the products produced *in situ* by *B. infantilis*, and the experiences of Ardoin<sup>1</sup> and Spiegelberg<sup>2</sup> certainly are in favor of this view.

#### SUMMARY.

(1) A spore-forming organism, *B. infantilis*, described above, has been isolated from each of a series of cases of infantilism.

(2) It has also been found in limited numbers in the feces of some although not all normal infants.

(3) *B. infantilis* is not an obligate intestinal bacillus, but a saprophytic organism which under certain undetermined conditions finds a suitable environment in the intestinal tract and proliferates there.

(4) It produces no agglutinins and there is no direct evidence indicative of its etiological relationship to infantilism.

(5) *B. infantilis* fed to a dog and a monkey caused in each animal a pronounced softening of the stools and diarrhoea. In the monkey, this diarrhoea was followed by a decided diminution in the Gram-negative gas-producing bacilli of the coli-aërogenes type, and a noteworthy increase in the Gram-positive acidophilic flora. *B. bifidus* in moderate numbers and *B. acidophilus* in excessive numbers were the dominant organisms. There was a gradual return to the normal type of stool, both macroscopically and microscopically.

(6) These experiments furnish evidence in favor of the view that the diarrhoea observed in cases of infantilism may be caused by irritant metabolic products resulting from the proliferation of *B. infantilis* in the intestinal tract.

In conclusion the writer wishes to express his indebtedness to Dr. C. A. Herter not only for the opportunity of studying these cases but also for many helpful suggestions and advice during the progress of the work. The photographs which accompany this paper were made by Dr. Leaming of the Rockefeller Institute, to whom the writer is indebted.

<sup>1</sup> *Loc. cit.*

<sup>2</sup> *Loc. cit.*

## EXPLANATION OF THE PLATES.

1. Pure culture of *B. infantilis*, showing Gram-positive and "punctate" forms, the latter, staining faintly. *a* shows an antennate form ( $\times 1000$ ).
2. Submerged colonies of *B. infantilis*, showing the floccose structure.
3. A typical field from an infantilism stool. The principal organisms represented are: *B. infantilis*, *B. acidophilus*, *B. bifidus*, *Mic. ovalis*, *B. coli*. The doubly contoured bodies, resembling large capsulated cocci are relatively common in typical infantilism stools. Their significance is unknown ( $\times 1000$ ).
4. A typical dextrose fermentation-tube sediment from a case of infantilism. I-IV, VI, *B. bifidus*; II, pseudo-branched form of *Mic. ovalis*; III, club-shaped form of *B. bifidus*; V, antennate form of *B. infantilis* ( $\times 1000$ ).

The diagnosis of these organisms is based upon the cultural study of the stool from which the photograph was prepared.

PNEUMOTHORAX AND POSTURE.

By CHARLES A. ELSBERG, M.D

## PNEUMOTHORAX AND POSTURE.<sup>1</sup>

By CHARLES A. ELSBERG, M.D.

(From the Department of Physiology and Pharmacology of the Laboratories of the Rockefeller Institute for Medical Research.)

The danger from the sudden entrance of air into the normal pleural cavity has, more than anything else, impeded the progress of the surgery of the lungs and of the other intrathoracic viscera. Because of the importance of the subject, much careful experimental and clinical work has been done, and many investigations have been made to determine the cause of the dangerous respiratory embarrassment which is apt to ensue. Little or no attention has, however, been paid to the question whether posture has any influence upon the symptoms which appear when the normal pleural cavity is laid open. In fact, this phase of the subject is mentioned in only a few publications. In a discussion on the surgery of the lungs at the congress of the French surgeons in 1906, Dépage<sup>2</sup> stated that he thought it best to have the patients in whom the pleural cavity had to be opened during the course of an operation flat on the abdomen. He did not, however, give any reasons why he recommended this position. Kocher,<sup>3</sup> in the last edition of his *Operative Surgery*, declares that, when the chest is to be opened, the patient should be placed on the side or abdomen, but he does not say whether he believes there is any advantage in the prone posture.

In this paper, experiments are described which were made with a view to determine what influence posture may have upon the symptoms of pneumothorax.

### EXPERIMENTAL INVESTIGATIONS.

*Open Pneumothorax.*—The following experiments deal with the subject of pneumothorax caused by a free opening into one or both

<sup>1</sup>Received for publication January 8, 1909.

<sup>2</sup>*Congrès de chirurgie, Transactions*, 1906, xix, 387.

<sup>3</sup>*Chirurgische Operationslehre*, 1907, p. 701.

pleural cavities. The method of operation for the open pneumothorax experiments was the following:

The animal was given a hypodermic injection of morphine several hours before the experiment was begun. Under ether anaesthesia, tracheotomy was performed, a tracheal canula inserted, and the administration of ether continued through the canula. A large skin flap was then raised on one side of the chest and a subperiosteal resection of one or more ribs made. The intercostal muscles were carefully divided and the parietal pleura was exposed. While the animal was deeply under the influence of the anaesthetic, a small opening was made in the pleura and the effect noted. The opening was then gradually enlarged. In a number of the animals, the pressure of the inspired and expired air was roughly measured by allowing the animal to breathe into a large bottle, the tube leading to the bottle from the tracheal canula being connected by means of a T-tube with a water manometer. During the entire time of observation the animals were kept deeply under the influence of the anaesthetic, so that all of the reflexes were abolished.

The symptoms which ensued, in my observations, when an opening was made into one or the other pleural cavity will not be described in detail. For the purpose of this paper, the following facts will suffice. When a small opening is made into one pleural cavity of a dog, air enters the pleural cavity with a hissing noise and the lung collapses. For a moment respiration ceases, then the breathing becomes more rapid and deeper and the animal makes violent efforts at respiration. If the opening in the chest be a small one (1 to 5 mm.), one of the following several conditions will ensue: (1) After a few moments of violent respiration, expiration becomes prolonged and the heart suddenly stops beating; (2) more often the breathing will gradually return to the normal although the amount of inspired and expired air will be found to be considerably below the normal; (3) sometimes, after a few minutes, the breathing becomes irregular, violent inspiratory efforts alternate with prolonged slow expirations, the heart's action becomes slow and irregular until it ceases altogether.

Many dogs stand a small opening into the pleural cavity well. The larger the opening, however, the more likely is the occurrence

of serious interference with respiration. In almost all animals in which the size of the opening approached to or exceeded that of the diameter of the animal's trachea, dyspnea and death followed. No matter how slowly and cautiously the opening was made, typical dyspnea of a preponderant expiratory character ensued, the heart became irregular and weak, the pleural septum between the two sides bulged into the opening in the chest with each violent expiration, and rupture of the septum with double pneumothorax or sudden stoppage of the heart occurred.

The statements of most of the experimenters agree with those above given. Most of their animals died from the pneumothorax when a large opening was made in the chest. Thus Gluck lost all of the dogs he used; Biondi lost four of five; Block, Marcus, Schmidt, Pourrat and Rodet, Quenu and Longet, Sauerbruch,<sup>4</sup> and many others had similar experiences. It should be mentioned that in the experiments of the above named authors the animals were operated upon in the supine position.

*The Influence of Posture.*—(a) *Supine and Lateral Postures.* Openings were made in the thorax, in various places on the right and left side, near the sternum, the vertebral column, the diaphragm or the clavicle. Here again the opening was at first of small size, then it was gradually enlarged. During the observations, the posture of the animal was frequently changed, so that the animal lay sometimes on its left side, on its right side, on its back, on its abdomen; sometimes its head was raised until the animal was almost suspended vertically, sometimes it was hung up by its hind legs. In all animals, excepting those that were kept on the belly, the symptoms which ensued when a small or large opening was made in the chest were those of open pneumothorax as described above. In the experiments, no differences were found between the symptoms produced by an opening in the right as compared with one in the left pleural cavity. Twenty dogs were used in these experiments; nineteen of the animals succumbed under the typical pneumothorax symptoms when a large opening into one pleural cavity was made. One dog, however, remained alive in spite of the

<sup>4</sup> A complete account of the work that has been done will be found in the paper of Sauerbruch, *Mittel. aus den Grenzgebieten der Med. und Chir.*, 1904, xiii, 399.

fact that a large opening (6 cm. in diameter) had been made in the right chest. The animal presented many of the typical symptoms of pneumothorax, but its heart action remained regular, and respiration—though irregular and weak—continued. There are some few dogs that are apparently quite insensitive to the entrance of air into the pleural cavity.

The foregoing statements may be illustrated by the following two abbreviated protocols.

EXPERIMENT 1.—Large black male. *On back*; deep anæsthesia; canula in trachea connected with large bottle and by T-tube with water manometer. Inspiration 40–50 mm., expiration 50 mm. The animal was observed for five minutes, during which time the parietal pleura was exposed in the usual manner. With a small knife an opening 4 mm. in length is made in the right pleura. Inspiration and expiration at once become violent; inspiratory pressure 4–8 mm., expiratory pressure 15–25 mm. The breathing becomes more and more labored and expiration begins to be stronger than inspiration. After three minutes, inspiration 2–5 mm., expiration 25–40 mm., the animal presents all of the symptoms of pneumothorax dyspnea. Respiration irregular, expiration prolonged, heart irregular and slow, marked bulging of mediastinal septum at each expiration. The inspiratory oscillations of the manometer are almost nothing. The heart suddenly stops beating.

EXPERIMENT 2.—Large male. *On back*; preparation as above in Experiment 1. The left pleura is exposed by resecting two ribs. Respiration 56; pulse 108. With a small knife, an opening measuring 3 mm. is now made. The animal stops breathing for 30 seconds, then breathing begins again at the same rate as before. Pulse now 136. Breathing is somewhat more labored. The animal is now turned *on its right side*; character and rate of respiration remains the same. No change is observed when the animal is turned on its *left side* or when turned *on its back* again. The opening in the pleura is now enlarged to 7 mm. Inspiration and expiration become deep and irregular; then inspiration becomes weak while expiration becomes more forcible and prolonged. With each violent expiratory effort the mediastinal septum bulges into the opening in the chest. Finally the septum ruptures and collapses, the heart then stops suddenly.

(b) *Prone Posture*.—In the following experiments, the animals were operated upon in the prone position, or they were first on the back and were later turned on the belly.

When a small opening is made in the pleural cavity of a dog that is lying on its belly, the animal will, in most instances, continue to breathe quietly like a normal animal. Even a very large opening (2 to 6 cm. in diameter) can be made, and breathing go on regularly and quietly, almost if not quite like the normal. Even one-

half of one chest wall can often be removed and the dog survive for hours. These statements are given after observations made in a large number of experiments, of which only the following few abbreviated protocols are given. It must be expressly stated that deep anæsthesia is essential for the success of the experiments.

EXPERIMENT 3.—Large brown bitch. *On belly*; tracheotomy; usual preparation.

4:40 p. m. Deep anæsthesia, inspiration 90 mm., expiration 70–90 mm.

4:50. Opening 3 mm. in diameter in left pleura, inspiration 40–60, expiration 50–70; animal is breathing quietly.

4:55. Opening enlarged to 10 mm., inspiration 30–40, expiration 40–50.

5:00. Opening enlarged to 2 cm., inspiration 25, expiration 30–40.

5:10. Opening enlarged to 4 cm., inspiration 20–25, expiration 30–40.

5:20. Inspiration 35–40, expiration 40–50.

5:30. Opening enlarged to 6 cm., inspiration 30, expiration 40.

5:40. Inspiration 40, expiration 40.

During the entire time of observation the animal breathed quietly and there was no evidence of dyspnea, in spite of the fact that the amount of inspired and expired air was about one-half of the normal.

EXPERIMENT 4.—Small black female. Under artificial respiration a large opening 4.5 by 7 cm. was made in the right chest while the dog was *on the belly*. Artificial respiration then stopped. The animal continued to breathe quietly and the heart action remained strong and regular for 45 minutes, when the observation ended.

EXPERIMENT 5.—Large brown male. With the animal *on the belly*, an opening 6 by 4 cm. was made in the left pleural cavity under artificial respiration. The animal continued to breathe quietly and regularly for over an hour. It was then killed.

If a dog, *on its back*, with an opening in its chest and with typical dyspnea due to the pneumothorax, is *turned on its belly*, the breathing will often become regular and quiet again, and the pressure of air breathed in and out will be found to be several times as great as when the animal was on its back. On the other hand, a dog *on its belly* with a large opening in one pleural cavity and breathing quietly, can be brought into a condition of grave dyspnea and asphyxia by *turning it on its back*. Sometimes it is even possible to resuscitate an animal that has stopped breathing by thus turning it on its belly.

This is illustrated by the following protocols:

EXPERIMENT 6.—Large black male. After the usual preparation (tracheotomy, canula connected with manometer, deep anæsthesia) the animal is placed *upon its back*, and an opening 4 mm. in diameter is made in the right

pleural cavity at 3:15 p. m. Respiration at once becomes deeper and expiratory dyspnea begins. Inspiration 90, expiration 100.

3:18 p. m. Inspiration 50, expiration 80.

3:20. Inspiration 20, expiration 90; violent dyspnea.

3:21. Opening in chest enlarged to 1 cm.; inspiration 20, expiration 90; dyspnea very violent.

3:22. Opening enlarged to 3 cm., inspiration 20, expiration 30.

3:22-½. Inspiration 5, expiration 10; heart action very irregular.

3:23. Inspiration 0, expiration 0; violent efforts at respiration, but manometer shows that no air is inhaled or exhaled. The heart suddenly stops beating. The animal is now quickly *turned on its belly* and artificial respiration given for one minute.

3:25. The animal is now breathing better again; inspiration 30 mm., expiration 40 mm., heart beating regularly.

3:26. The animal is now *turned on its back*. Dyspnea begins at once; inspiration 0-5, expiration 5-10.

3:27. *Turned on belly*; inspiration 30, expiration 40; breathing somewhat stertorous at first, but becomes gradually quiet and regular.

3:35. Animal has been breathing quietly for several minutes; it is now *turned upon its back*. Violent dyspnea begins at once, and continues until rupture of the mediastinal septum occurs two minutes later. The animal cannot be revived by artificial respiration.

EXPERIMENT 7.—Large dog. *On back*; canula in trachea connected with water manometer; deep anæsthesia; inspiration 50 mm., expiration 40-50 mm. An opening 7 mm. in diameter is made in the left pleural cavity in the usual manner. The breathing becomes slightly labored and irregular. After 12 minutes the breathing is still good although there is now well marked expiratory dyspnea. Inspiration 20-35 mm., expiration 60-90 mm.

The board on which the animal is tied is now inverted so that the dog lies with its *belly downward*. The character of the breathing improves at once and the pressure of inspired and expired air is much greater; inspiration 130, expiration 140; at times inspiration 50-70, expiration 80-120; sometimes inspiration is more deep than expiration. Breathing good though somewhat labored. The board is now turned over so that the dog lies *on its back again*; violent dyspnea begins at once. The opening in the chest is now quickly enlarged to 3 cm.; inspiration and expiration are now almost nil although the animal makes violent expiratory efforts; pulse slow and weak. The board is now quickly turned over again so that the animal hangs *belly downward*; breathing becomes better and heart action stronger. Especially noticeable is the fact that the mediastinal septum no longer bulges on expiration. A few minutes later, the board is turned again so that the dog is *again on its back*; with one violent expiratory effort the mediastinal septum ruptures and the heart stops.

A dog on its back will sometimes stand a double pneumothorax when the opening in each pleura is a very small one—not more than 1 to 2 mm. in diameter—and made very slowly. But with the dog on its belly, an opening almost 1 cm. in diameter can sometimes be made, if cautiously done, and the animal may continue to breathe

for a considerable time—sometimes for more than one hour. This was demonstrated by the following experiment:

EXPERIMENT 8.—Large brown male. *On back*; usual preparation; inspiration 140 mm., expiration 140 mm. Under deep anæsthesia; inspiration 100–140, expiration 90–100 mm. An opening 3 mm. in diameter is now made in the left pleura; inspiration 90, expiration 90. Five minutes later; inspiration 70–85, expiration 80–90; there is slight dyspnea. An opening 3 mm. in diameter is now made in the right pleura; the dog awakes from the anæsthesia and begins to struggle; inspiration 60–80 mm., expiration 100–130 mm. The animal is again deeply anesthetized; inspiration 50 mm., expiration 90 mm.; moderate expiratory dyspnea. The animal is now *turned on its belly*; inspiration and expiration remains the same, but the dyspnea is distinctly less marked. Five minutes later there is only slight expiratory dyspnea. The opening in the left pleura is now carefully enlarged to 1 cm. There is no essential change in the character of the respiration. The animal is now turned upon its back; expiratory dyspnea begins at once, heart action slow and irregular; inspiratory and expiratory pressures 0 mm. After three minutes of artificial respiration; inspiration 2–3 mm., expiration 5–15 mm. The animal is now quickly *turned upon its belly*; it is at once evident that there is much less dyspnea; inspiration 20–30 mm., expiration 30–40 mm. During the following thirty minutes the animal continues to breathe well without more than slight dyspnea.

*The Relation of the Position of the Heart to the Influence of Posture.*—It has been proved by the above experiments that there is a decided difference between the symptoms of pneumothorax in dogs operated upon in the prone as compared with those in the supine position. Not only did the animals present few changes in respiration and heart action when they were on the belly, if a small opening was made in the pleura, but very large openings could be made without causing serious symptoms, if the animals were kept on the belly or were turned into that position. The measurements of the pressure of inspired and expired air showed that decidedly more air was drawn in and exhaled when the animal was on its belly.

This is not due to the animal's lying on its chest, because if the animal is suspended with its belly downward (see Experiment 7) so that no pressure is made on the thorax, similar effects are to be observed. It might be due, however, to the change which occurs in the position of the heart in the thorax in the change from the supine to the prone position. When the animal lies on its back, the heart falls backward toward the vertebral column; when the animal lies upon its belly, the heart falls toward the anterior chest wall. In order to determine whether this change in the position of the heart

had anything to do with the difference in the pneumothorax symptoms, the organ was fixed to the anterior chest wall or to the vertebral column, and the effect of posture upon the symptoms in these animals was studied.

Under artificial respiration and ether anæsthesia, a small opening was made in one pleural cavity, the pericardium grasped with a forceps and brought into the wound. If the animal was operated upon in the supine position the opening in the pleural cavity was made near the sternum. In this animal, the pericardium was attached to the anterior chest wall near the sternum by a suture which passed around a costal cartilage at the sternum. If the animal was operated upon in the prone position, the pericardium was attached to the vertebral column by a suture which passed from the pericardial sac through the muscles of the back near the vertebræ. The ends of the sutures in both cases were left long so that they could be easily cut when desired. In this manner, in the animals that were operated upon while on their back, the pericardial sac and heart were pulled forward against the anterior chest wall; in the animals operated upon while on their belly, the pericardial sac and heart were pulled toward the vertebral column. After the suture had been tied, artificial respiration was stopped, and the opening in the pleural cavity enlarged. The animal was observed for a time and then the suture which fixed the heart was cut. The results of these experiments can be summed up as follows:

In the case of the dog lying *on its back* with the heart attached to the anterior chest wall so that it could not fall backwards, a large opening could be made in one pleura—almost as large as in former animals lying on the belly—without the occurrence of serious respiratory difficulty. In other words, when the heart is fixed to the anterior chest wall, a fairly large opening can be made in the pleural cavity and breathing remain good no matter what the posture of the animal. The moment, however, that the stitch was cut and the heart allowed to drop backward, the typical pneumothorax symptoms occurred.

In the case of the dog *on its belly* with the pericardial sac attached to the posterior chest wall by a suture, no more than a very small opening could be made in one pleura without the immediate appearance of pneumothorax symptoms. In other words, if the

heart is fixed to the posterior chest wall, pneumothorax symptoms appear as soon as there is an opening into the pleural cavity no matter what the posture of the animal. If the dog is lying on its belly, and the stitch is cut so that the heart can fall toward the anterior chest wall, the symptoms disappear or are much relieved, and the animal then acts as would any dog lying on its belly with an opening in one pleural cavity. Although there were some exceptions, the experiments were successful in a sufficient number of cases to justify the conclusion that the position assumed by the heart, in the prone posture, is an important factor in the difference between the symptoms produced by an opening in the pleural cavity in the prone as compared with the supine animal.

#### DISCUSSION AND SUMMARY.

The last mentioned fact may perhaps find its explanation in the following statements: the two pleural cavities are separated by the layers of the anterior and posterior mediastinal septa. Between the two lies the heart. In the dog, the posterior seems to be somewhat tougher than the anterior septum, and somewhat more fixed and tense. With violent respiratory movements, it is the anterior septum which more especially flaps to and fro and bulges when an opening in the pleura has been made, and it is the anterior septum which is so apt to rupture and thus cause double pneumothorax and the death of the animal. When the dog is on its back, the heart falls backward and the bulging of the anterior mediastinal septum is made more easy. It is different when the animal is on its belly. The heart falls toward the anterior chest wall and thus supports the anterior septum; hence the flapping of the septum, the interference with the respiration of the lung on the sound side, the bulging on expiration on the open side, can not so readily occur.

The danger of the open pneumothorax is greatly lessened when the animal is in the prone position. In the supine position the danger of the pneumothorax is due to the falling back of the heart and thus facilitating the rupture of the fragile anterior mediastinal septum; the danger is therefore obviated by fixing the pericardium to the anterior wall of the thorax.

I desire to state that I am greatly indebted to Dr. S. J. Meltzer for much aid and many suggestions.





AN EXPERIMENTAL STUDY OF THE INFLUENCE OF  
KIDNEY EXTRACTS AND OF THE SERUM OF  
ANIMALS WITH RENAL LESIONS UPON  
THE BLOOD PRESSURE.

By RICHARD M. PEARCE, M.D.

## AN EXPERIMENTAL STUDY OF THE INFLUENCE OF KIDNEY EXTRACTS AND OF THE SERUM OF ANIMALS WITH RENAL LESIONS UPON THE BLOOD PRESSURE.<sup>1</sup>

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The question of the influence of kidney extracts on the blood pressure is one which is intimately associated on the one hand with the theory of the internal secretion of the kidney, and, on the other, with the problem of the relation between cardio-vascular disturbances and renal disease. The first experimental contribution to this subject was that of Tigerstedt and Bergman (1) who offered evidence of the presence of a pressor substance in extracts of the kidney of the rabbit. This substance, which they named "renin" they regarded as an internal secretion passing normally into the blood.

Several theories, based on this principle of internal secretion, have been formulated by clinicians to explain the vascular disturbances associated with chronic nephritis. It is sufficient to mention Forlanini and Riva-Rocci (2), who hold that a blood-pressure-raising substance is formed in increased amount in the diseased kidney; and Shaw (3), who attempts to offer a basis for Traube's theory of uræmia by assuming that the substance causing arteriospasm, and thus producing cerebral disturbances without postmortem lesions, is possibly the pressor substance "renin."

Last year during the preparation of a discussion (4) of this phase of renal activity, it became necessary, on account of the confused state of the literature, to repeat these experiments, in order that the subject might be properly presented. The few experiments

<sup>1</sup> This investigation, aided by a grant from the Rockefeller Institute for Medical Research, was begun in the Bender Hygiene Laboratory, Albany, N. Y., and completed in the Carnegie Laboratory of The University and Bellevue Hospital Medical College, New York. Received for publication January 5, 1909.

made at that time offered no support of the theory that a pressor substance exists in the normal kidney of all animals and was therefore of little value as a basis for the consideration of the pathology of circulatory disturbances. As some of the results were, however, of physiological interest I have, during the past few months, again taken up certain phases of the general problem. The results of these investigations and also of a study of the influence on blood pressure of the serum of animals with various forms of experimental injury are here presented.

The literature is brief and may be summarized as follows:

The pressor substance of the rabbit's kidney described by Tigerstedt and Bergman in 1898 was obtained from the cortex of the kidney and was not present or was present only to a slight extent in the medulla. This substance could be extracted from the fresh organ by salt solution, alcohol, fresh blood and, to a less extent, by cold water. Extracts prepared by boiling gave no effect. The substance was non-dialysable and the investigators therefore concluded that it could not be any of the salts of the urine. To this substance they gave the name "renin" and they regarded it as an internal secretion of the kidney normally passing into the blood. The rise of pressure, which varies from a few millimeters to 25 or 35 mm. Hg, they believed to be due to an action on the peripheral nerve centers as well as possibly on the spinal cord. Very small amounts caused as much effect as larger doses and each repeated injection produced an effect as great as the first injection.

In order to demonstrate the presence of this supposed pressor substance in the circulating blood, they injected blood from the renal vein into the vessels of rabbits which had suffered double nephrectomy. A moderate rise followed. For example, in one animal, twenty-four hours after nephrectomy a rise of 18 mm. Hg occurred. These last experiments were repeated by Lewandowsky (5) the following year, and although Tigerstedt's rise of pressure was confirmed, Lewandowsky obtained a similar transient pressor effect in his controls by injecting blood from the general venous (jugular vein) and arterial systems. The rise he considered to be due in part to the rapidity of injection, but as the same rise was not obtained by injections of salt solution, he concludes that the effect

is due to some substance or substances in defibrinated blood capable of pressor effect and not to an internal secretion of the kidney.

Lewandowsky's results appear to have discouraged the further extensive investigation of Tigerstedt's observations which one would have expected. The only references in the literature, at least as far as I have been able to determine, are those of Livon (6), Fiori (7), Vincent and Sheen (8), and Shaw. Of these the first two describe a pressor effect and Vincent and Sheen observed various results, sometimes a fall, sometimes a rise in pressure, and occasionally no effect. Shaw, using the cat, obtained almost uniformly striking and prolonged rise in pressure, varying from 1 to 72 mm. Hg. Mention must also be made of the observations of Oliver (9) published a year before those of Tigerstedt. Utilizing the exposed mesentery of a frog to determine the effect of various organ extracts upon the peripheral vessels, he constantly obtained with the adrenal extract a most decisive contraction but no invariable effect with extracts of the kidney or various other organs.

When one analyzes these various observations there is some evidence to indicate that the result may depend on whether or not the kidney extract was injected into an animal of the same species. Thus Tigerstedt, in a series of about fifty experiments with the rabbit, obtained almost uniformly a pressor effect, as did also Shaw—using the cat—in all but one of nineteen injections.<sup>2</sup> Vincent and Sheen's injection were not always into animals of the same species and their results varied. Livon does not give the details of his work, but his results were uniform, as were also those of Fiori, who used the rabbit.

The determination of the difference between the effect of homologous and of heterologous injections has been one of the more important phases of the present investigation.

*Methods.*—The extracts were prepared as follows: (a) *Salt solution extract.* A weighed portion of kidney cortex was rubbed up in a mortar with the aid of powdered glass. Salt solution (0.85 per cent.) in the proportion of two parts to one of kidney was then added and the grinding continued. The mixture was then squeezed

<sup>2</sup> In a second series of experiments Shaw's results were not so uniform and he came to the conclusion that much depends on the form of anæsthesia.

through several layers of gauze and the resulting fluid filtered through paper. The fluid thus obtained was blood stained, usually opalescent, though occasionally clear; microscopically, it contained innumerable fine granules. (b) *Dried filtrate of alcoholic extract dissolved in salt solution.* A weighed portion of finely divided kidney cortex was rubbed up with absolute alcohol and filtered; the filtrate was evaporated to dryness at room temperature and to this was added an amount of salt solution equal to twice the bulk of the original kidney substance.

This latter extract was the one used most frequently by Tigerstedt and Bergman, presumably on account of the ease with which it could be kept without deterioration. They state, however, that the salt solution extract is of equal value, and as I found this to be true, and also as it seemed more desirable to use fresh extract, it is this extract that I have employed in the majority of my experiments. Extracts prepared by boiling and with glycerine have been used only as controls.

It must be admitted that the salt solution method of extracting is crude, but as I found, in control observations, that by its use a very active extract of the adrenal could be obtained, I have considered the method a satisfactory one.

All kidneys used in preparing extracts were obtained from animals sacrificed for that purpose, thus eliminating the influence of products formed during decomposition. The extracts were tested usually within one hour, and seldom later than two hours after obtaining the kidney substance and in the meantime were kept on ice as much as possible. The fluid in the earlier experiments was warmed before injecting to about 35° C. but as controls showed that temperature was a negligible factor, the fluid in the later work was used at room temperature. The pressure was taken in the carotid artery; the extract to be tested was injected as a rule into the saphenous vein or frequently, in the case of the rabbit, in the ear vein. All animals were anæsthetized with ether, except that in some instances rabbits were given urethane in the peritoneal cavity in doses of .025 grm. per kilo of body weight. The usual dose of kidney extract was two or three cubic centimeters, though it varied from one to five cubic centimeters in some instances.

*Effect of Rabbit's Kidney Extract on the Rabbit.*—Nine extracts of kidney prepared from as many rabbits were tested on nine different animals, with a total of eighteen injections. Two injections caused no change in pressure, but in the other sixteen there was a slight rise with rapid return to normal. In three of the latter a faint initial fall was observed and in two a drop followed a rise. The rise was never more than twelve and usually only 6 to 10 mm. Hg. In order to control the effect of substances in the blood, two of these extracts were made from kidneys which had been freed from blood by perfusion with salt solution. The action of these extracts did not differ from that of those made from the blood-containing kidney.

As the disturbance of pressure was very slight, a series of controls was undertaken. This series included the injection of two to three cubic centimeters of extracts of the rabbit's liver, rabbit's urine, rabbit's blood serum, Locke's solution and salt solution. Each of these was tested three times and caused almost constantly the same effect as the kidney extract. The only exceptions were absence of change in pressure in two instances when rabbit's serum and liver respectively were injected. These results appear to indicate that in an animal, as the rabbit, whose circulation is easily disturbed, the effect is largely, if not entirely, mechanical.

This conclusion is supported by further experiments in which attempts were made to remove the pressor substance. It was shown that boiled and filtered extracts, as well as the dialysate of the extract, produced the same slight transient rise of pressure as did the original kidney extract. Similar results were obtained with various alcoholic extracts, with a salt solution extract twenty-four hours old, with an extract twenty-four hours old which had been kept at a temperature of  $37.5^{\circ}$  for the last four hours of that period, and also with extracts of rabbit's kidneys which had been deprived of their blood supply by ligation for four hours.<sup>3</sup> Disappearance of the pressor substance was evident only in obviously putrid extracts, four or more days old; such extracts caused an immediate decided drop in pressure.

<sup>3</sup> These extracts caused an initial rise, which was, however, always followed by a moderate and prolonged drop in pressure.

*Effect of Dog's Kidney Extract on the Dog.*—This portion of the investigation has to do entirely with the study of a depressor substance of the dog's kidney. Salt solution extracts of twelve different kidneys were tested on as many dogs, with a total of twenty-nine injections. Five of the extracts caused an immediate and decided drop of 58 to 96 mm. Hg with a slow return to normal; one caused a moderate drop of 26 and one a very slight drop of 14 mm. Hg.

Three of the extracts caused a fall in pressure which ended in death. Of this I can offer no explanation; the result is apparently analogous to that observed by Pemberton and Sweet (10) after the injection into the dog of extracts of the central nervous system of the same animal.

In no instance was a rise of pressure observed, and second and third injections always produced an effect equal to the first injection. Boiled and alcoholic extracts had less effect than salt solution extracts but still possessed a very definite depressor action. It is noteworthy that the alcoholic extract was less active than the boiled extract. Only by dialysis could the depressor substance be removed; but while the dialysate contained an active depressor substance the original fluid had no pressor effect. Extracts of ligated kidneys showed no diminution in depressor action.

As the various methods of removing the depressor substance, such as those utilized by Schäfer and Vincent (11), Osborne and Vincent (12) and Joseph (13), failed to demonstrate a coexistent pressor substance, this phase of the work was abandoned.

Control injections demonstrated that dog's serum, defibrinated blood, or liver extract has no effect or a slight depressor effect while fresh urine of the dog has a decided depressor effect equal to that of the kidney extract.

These observations indicate that the depressor action of the kidney extract is due to the presence of the salts (potassium?) of the urine. This view is supported by the dialysis experiments and also by the fact that an extract of washed kidney, from which urine is presumably more or less removed, has less depressor power than an extract from the opposite unwashed kidney of the same animal.

The possibility that the depressor substance was cholin was con-

sidered, but all attempts to demonstrate cholin in the extracts by the methods described by Donath (14) were unsuccessful.

*Effect of Cat's Kidney Extract on the Cat.*—These experiments are few in number and were undertaken mainly for comparison with the effect on the dog of the dog's kidney extracts and of the urine. Shaw has demonstrated very conclusively the pressor effect of extracts of the cat's kidney and his results I have readily confirmed, though one of four extracts used caused a fall in pressure. The rise in pressure varies from 22 to 34 mm. Hg. The greater rise of 72 mm. Hg which Shaw describes I have not observed. This pressor effect is not a property of the cat's liver. On the other hand, the cat's urine, like that of the dog, causes a decidedly sharp drop in pressure; the lowest observed being 76 mm. Hg.

These observations therefore prove decisively that a kidney may contain a pressor substance which cannot be masked by the depressor effect of the urine, a point which could not be determined absolutely for the dog's kidney.

*Heterologous Injections.*—In the above experiments practically constant results have been obtained when the kidney extract has been injected into an animal of the same species as that furnishing the kidney; that is, in the rabbit a slight rise, in the cat a moderate rise and in the dog a decided fall in pressure. Cross injections made to determine the constancy of this action have given somewhat surprising results which are difficult of interpretation. An extract of the rabbit's kidney injected into the dog gives, not a rise as it does in the rabbit, but a very definite fall in pressure. This has been observed in four animals receiving four different kidney extracts. In one the drop was only 20 mm. but in the other three it was 58, 60 and 64 mm. Hg.

On the other hand, the dog's kidney extract, which in the dog produces a great fall in pressure, when injected into the rabbit causes a slight rise. This rise differs in no way from that which occurs when the rabbit receives rabbit's kidney. This has been observed in five rabbits receiving extracts of six different dog's kidneys. Likewise the dog's urine, which has a decided depressor effect on the dog, fails to act in this way on the rabbit but causes a barely perceptible transient rise of from 4 to 6 mm. Hg.

These results are difficult to explain. As far as the two animals in question are concerned each reacts to the other's kidney substance in the same way that it does to its own, even though the particular kidney substance may have had the opposite effect on the animal from which it was taken. In this way the action of kidney extracts differs from that of adrenal extracts which have a constant effect on many, if not on all animals. Whether this principle would hold with other animals is an interesting question. In this connection it is worthy of note that Brown and Joseph (15) in their study of the effect of organ extracts of cold-blooded animals on warm-blooded animals found that shark's kidney injected into the dog caused a rapid fall of 80 mm. Hg.

As the interest lay chiefly in the demonstration of pressor substances, this phase of the subject has not been further followed. The evidence is, I believe, sufficient to indicate that a pressor substance is not constantly present in all kidney extracts.

*The Effect of Extracts of Kidneys with Experimental Nephritis.*—The object of these experiments was to determine whether or not extracts of the kidney of experimental nephritis had an increased pressor activity. The kidneys were obtained from rabbits on the third to the fifth day of chromate or uranium poisoning, at a time when the urine contained an abundance of albumin. In no instance did such extracts have an effect different from those prepared from normal rabbit's kidney.

*Effect of the Blood Serum of Animals with Lesions of the Kidneys.*—If it is assumed that disease of the kidney leads to the retention of toxic products affecting blood pressure it might be possible to demonstrate these substances in the serum of animals whose kidneys had been injured in various ways. I have conducted a series of blood pressure experiments along this line. The influence of kidney reduction was first studied.

The recently published investigations of Pässler and Heineke (16) have aroused new interest in the experimental study of the relation of the kidney to increased blood pressure and cardiac hypertrophy. These investigators found that after the removal of a considerable portion of the kidney substance, approximately two-thirds to three-fourths, a rise of blood pressure occurred which

was permanent and associated with cardiac hypertrophy. This result was not constant, but occurred in about twenty-five per cent. of the animals which survived by at least four weeks a considerable reduction of kidney substance. It was observed that arterial spasm with further rise of blood pressure quickly followed stimuli which in normal animals would produce little effect. These observations suggest, according to Pässler and Heineke, that the heart hypertrophy is due to increased work resulting from the circulatory disturbances caused by tendency to arterial spasm, and that the vascular spasm is due in its turn to the effect of retained toxic substances. In the hope of demonstrating a pressor action by the serum of such animals, I have utilized the material of other investigations (17), which were carried on simultaneously with the one here described, and in which the substance of the kidney had been reduced by operation.<sup>4</sup>

Serum was obtained from five dogs; in four of these three-quarters of the kidney substance had been removed and the serum obtained five, thirty-four, thirty-nine, and fifty-six days respectively after the operation. The fifth lot of serum was that of a dog from which one-half of each kidney had been removed fifty-four days previously. The animals receiving the sera were under ether anæsthesia with cannula in the carotid artery; the serum was injected, in doses of twenty cubic centimeters, into the saphenous vein.

In each of the five animals thus treated a slight but definite drop in pressure, varying from 6 to 14 mm. Hg, occurred. In two animals the serum injection was repeated after several minutes had elapsed and in two others defibrinated blood was also injected. In all the same drop occurred. The injection of like quantities of

<sup>4</sup> Although no consistent effort was made to confirm the observations of Pässler and Heineke concerning heart hypertrophy, it is worthy of mention that in one of four dogs which lived at least five weeks after three-quarters or more of the kidney substance was removed, a definite cardiac enlargement was found. This was a dog with but one-seventh of its kidney substance remaining five weeks after the last operation. The animal gave a pressure in the carotid under ether anæsthesia of 160 and 175 mm. Hg as extremes of three observations within a half-hour period and at autopsy presented an obviously hypertrophied and slightly dilated heart. Three other animals living respectively forty-one, forty-eight and fifty-seven days showed no evidence of cardiac enlargement.

normal dog serum or of normal salt solution gave a slight rise or was followed by no appreciable change.

In like manner three dogs received the serum of animals with a spontaneous nephritis. This form of nephritis, which I have described elsewhere (18), was found in three animals during the course of this investigation. Serum was obtained from each dog and injected into another dog under the same conditions as described above. A very slight rise was obtained with one serum and an equally slight drop with the other two.

Both dogs and rabbits have been utilized in the study of the serum of experimental nephritis. A nephritis was produced by repeated subcutaneous injections of potassium chromate or uranium nitrate, and also, in the case of the rabbits, of arsenic. After three to four days, when the urine showed an abundance of albumin, the animal was bled and the effect of its serum upon the blood pressure tested on another animal of the same species. As far as I am aware the only other experiments of this type recorded are those of Ascoli and Figari (19) with nephrotoxic immune sera which I repeated several years ago with negative results (18).

The results of these experiments in the case of the dog have been rather surprising, for although each serum gives practically a uniform effect, one causes a fall and the other a rise in pressure. Thus each of four different uranium sera caused a prompt drop in pressure of from 14 to 42 mm. Hg (14, 28, 30 and 42 mm.) on initial injection and a drop of from 14 to 16 mm. on second and third injections. In no instance did this serum produce a rise or a doubtful effect. An equal number of chromate sera, on the contrary, uniformly caused a moderate but immediate rise in pressure. In one instance this was only 4 mm. Hg and represents perhaps the mere mechanical factor of injection; in the others the rise was 10 mm. in two, and 14 mm. in the fourth. A second injection in these animals caused a rise equal to the first, except in one animal which responded by a drop on the second, third and fourth injections, the drop varying from 10 to 26 mm. Hg.

Similar experiments with rabbits have given very conflicting results.<sup>5</sup> Chromate, uranium and arsenic sera give no uniform

<sup>5</sup> I wish to correct a statement in a previous (4) publication in which brief reference was made to these experiments with rabbits. It was stated that in

effect. Each may cause a rise or a fall in pressure, the fall, however, occurring three times as often as the rise. Chromate sera have given as extremes a rise of 14 and a drop of 30 mm. Hg; uranium, a rise of 38 mm. and a drop of 34 mm.; arsenic, a rise of 40 mm. and a drop of 30 mm. In all, however, the effect has been long continued, lasting for from twenty-four to forty seconds before producing maximum effect. In each instance the return to normal level has been correspondingly slow. In this respect this result with rabbits differs from the effect produced by analogous sera in the dog and from that produced in the rabbit by kidney extracts. The lack of uniformity, however, renders an interpretation impossible.

In connection with the serum injections in both dog and rabbit it may naturally be suggested that the effect is due to minute quantities of the original poison in the serum. The possibility of carrying over in the serum minute amounts of the salts injected must be considered, but it has been impossible to detect these salts in the filtrate of the serum concentrated after coagulation. The tests employed, however, are not so sensitive as entirely to exclude the persistence of these salts. On the other hand, if present, they would occur in such minute amounts that it seems improbable that they could have anything to do with the effect described. That the drop caused by uranium serum when injected into the dog is not due to traces of the uranium nitrate is shown by the fact that small amounts (0.0075 gr.) have no effect on the blood pressure, while larger amounts (0.0375 gr.) have a pressor effect. The effect of traces of chromate on the dog was unfortunately neglected. In connection with the experiments on rabbits it is noteworthy that the injection of uranium nitrate (0.0018 grm.), potassium chromate (0.0075 grm.) and arsenic (0.0025 grm.) caused a long continued fall in pressure varying from 6 to 14 mm. Hg. Although the fall is not so great as that due to the respective sera it detracts from the importance of the results with this animal.

That the effects described above are not due to substances in the normal sera or to the mechanical force of injection has been demonstrated in rabbits both chromate and uranium sera caused a rise in pressure and never a fall. This applied to experiments made up to that time and was justified by the earlier results of the work, but the results here presented show that both sera may cause a fall in pressure more frequently than they cause a rise.

terminated by injections of the serum of the respective animals and of equal amounts of salt solution. Such injections caused no change in pressure or a very slight transient rise according to the speed of injection.

Even if the experiments with rabbits are considered of doubtful value, the entire series of serum experiments, and especially those with dogs, suggests that disturbances of kidney function do cause the appearance of substances in the blood serum which have an effect on the blood pressure. Speculation concerning the nature of these bodies would be unprofitable but the variation in the dog is suggestive. Potassium chromate produces parenchymatous changes in the kidney, while uranium appears to affect both tubular and vascular structures. The difference in action of the two sera may possibly be explained by the difference in disturbance of kidney function in the animal furnishing the serum, and it is possible that more work along this line, especially with sera of chronic forms of nephritis, would yield valuable information. The presence in the serum of experimental nephritis of blood-pressure-disturbing substances is very suggestive in view of my recent study (20) of nephrotoxic substances in such serum.

#### SUMMARY.

1. Extracts of the rabbit's kidney injected into the rabbit cause a slight increase in blood pressure which is barely more than that due to the mechanical effect of the injection.

2. Extracts of the dog's kidney injected into the dog cause a decided fall in pressure; an equal fall may be caused by the dog's urine. A series of control experiments indicates that the fall caused by the kidney extract may be due to the urinary salts which it contains.

3. Extracts of cat's kidney cause a rise in pressure. As the cat's urine causes a fall, this rise in pressure indicates the possibility of a kidney extract containing a pressor substance which cannot be influenced by the depressor substance of the urine.

4. Rabbit's kidney, which in the rabbit produces a slight rise, when injected into the dog causes a drop comparable to that caused by the dog's kidney itself. Similarly, the dog's kidney, which in-

jected into the dog causes a drop, produces in the rabbit a rise analogous to that produced by rabbit's kidney. It is evident therefore that these pressor and depressor substances of the kidneys in question do not have a constant effect on all animals as do the extracts of the adrenal gland.

5. Extracts of kidneys which are the seat of various forms of nephritis cause the same effect as extracts of normal kidneys.

6. The serum of dogs with considerable reduction of kidney substance causes a slight fall in pressure; the serum of dogs with spontaneous nephritis gives divergent results, as does also the serum of rabbits with various forms of acute nephritis. The serum of dogs with chromate nephritis causes a slight rise, while that of dogs with uranium nephritis produces a sharp and decided fall in pressure. Although there is no uniformity in these results, their general character, and especially the experience with uranium and chromate sera of the dog, suggests that pressure-disturbing substances are present in the serum as the result of the kidney lesion. The very slight evidence of the constant presence of a pressor substance, however, offers little support to the theory that such a substance is furnished by the diseased kidney or is due to disturbances of metabolism caused by disease of the kidney.

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## THE EFFECTS OF ROENTGEN IRRADIATION UPON THE CHANGES IN THE CELL CONTENT OF THE BLOOD AND LYMPH INDUCED BY INJECTIONS OF PILOCARPINE.<sup>1</sup>

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The work upon which this report is based, is practically a continuation of that done by Dr. F. Peyton Rous, in this laboratory, during the last two years. Rous (1) gives a preliminary review of the experimental work done in relation to the blood changes induced by pilocarpine, and also refers to the work of Lefmann and Gasis in determining the effect of Roentgen irradiation on these changes. He also appends to his report a complete bibliography of the subject, which has been very useful in reviewing the work done under this general topic. His work had to do, particularly, with the influence of pilocarpine on the cell content of the thoracic duct as ascertained by making counts and estimations based upon the output of the duct by way of an established fistula. From his experiments he was led to state that the intravenous injection of pilocarpine nitrate causes in the dog so rapid and extensive an increase in lymphocytes in the output by way of the thoracic duct as to explain a large part, if not all, of the lymphocytosis shown in the blood of the animal under treatment with this drug. He also confirms, by calculation based upon the total amount of blood in circulation and the cell content and rate of flow of the lymph from the thoracic duct, the conclusions of previous workers (Biedl and Decastello (2), Selinoff (3), Crescenzi (4), Parodi (5)), that the thoracic duct is the principal avenue by which the lymphocytes pass from the lymphocyte-forming organs to the general blood circulation. The various theories as to how this lymphocytosis might be

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brought about are considered critically in the body of his paper, and are left without any definite conclusion, but there is shown an inclination toward the idea of Harvey (10), *i. e.*, that the phenomenon is mainly a consequence of contraction of the smooth muscle of the spleen and lymph glands.

Before undertaking the experimentation immediately necessary to determine the point indicated by my topic, it was thought best to make a few preliminary tests in order to confirm some observations which have already been given out, also to fill out some small gaps existing in the efforts to arrive at the effects of pilocarpine on the blood elements and on the input of lymphocytes by way of the thoracic duct. The increase in white cells in the circulation after administration of pilocarpine has been so definitely established as to render it practically superfluous to repeat the observation (Horbaczewski (6), Ruzicka (7), Waldstein (8), Lefmann (9), Harvey (10), Gasis (11), Rous (1)).

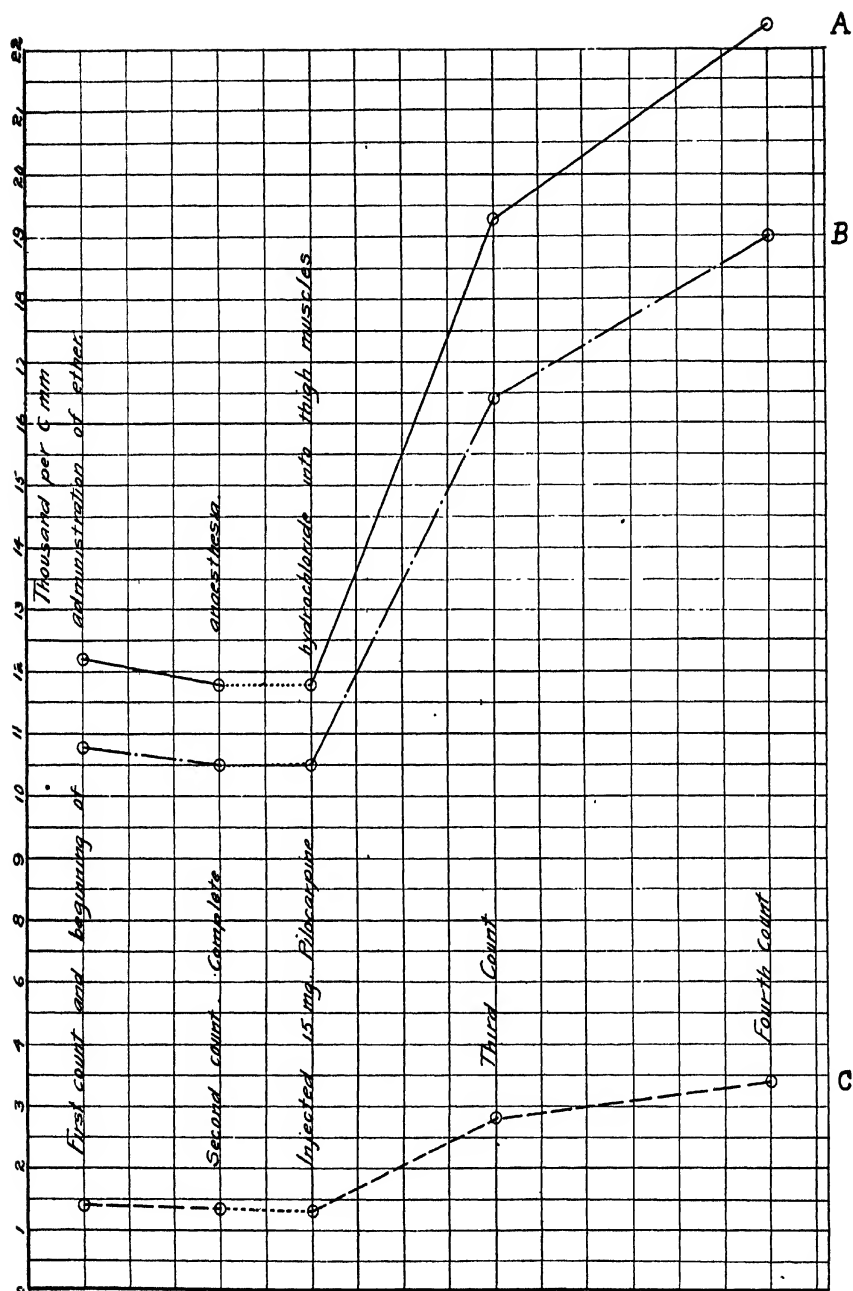
In connection with Rous's report, I may refer to an unpublished piece of work done a year ago in this laboratory by T. Dewitt Gordon. In working with pilocarpine on rabbits, he secured a great increase in white cells in the blood, characterized especially by the increase in the polymorphonuclear type of cells. As this did not seem to accord with previous tests, and especially as the rabbit can not be considered a satisfactory animal from whose blood changes any definite conclusions should be made, also because his blood counts were made with intervals of days, during which time other elements might have entered into the case so as to affect the proper changes, his work was not carried further and he made no further trials on other animals. It suggested to me, however, the fact that the "leucocytosis affecting the polymorphonuclear elements" which Rous reports as accompanying the lymphocytosis evident in his findings does not bear such a relation to the operations on the animals as to warrant attributing it at all to the surgical operations. This is clearly shown by an examination of the changes secured by Rous which I have taken the privilege to plot and describe here as Curves I, II and III.<sup>2</sup>

<sup>2</sup> In view of the detailed descriptions of Curves I, II and III these curves have been omitted.

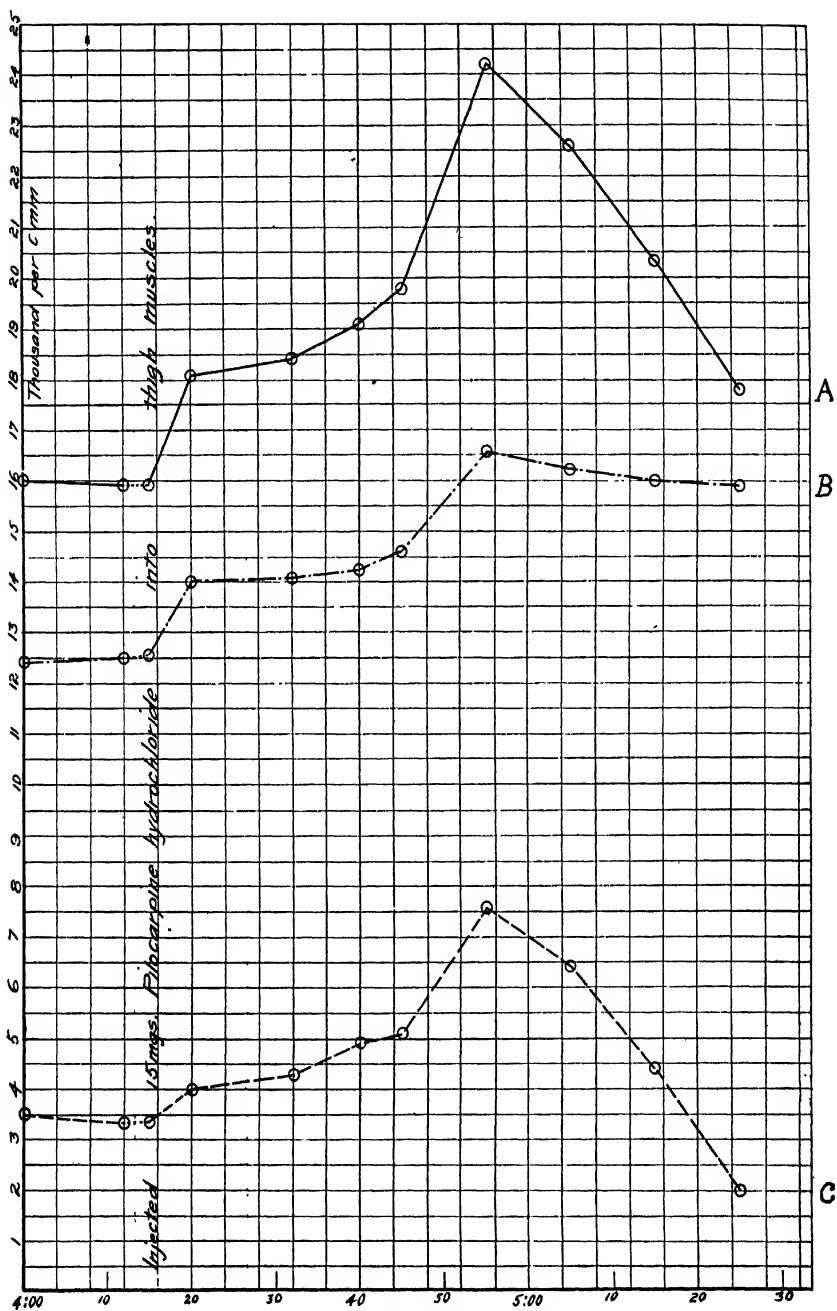
*Curve I.*—From this it is seen that the operation—exposure of the left external jugular vein and insertion of a cannula—was done at 9:50 o'clock. The first cell count was made at 10:15, and a second count was taken at 10:45. Now during this half hour the absolute and relative numbers of the various forms remained practically constant. The figures taken literally show a slight decrease in polymorphonuclears which, however, is so small as to be negligible. Then at 10:50 o'clock 20 mg. of pilocarpine nitrate were injected into the vein, and at the end of the next half hour the third count was made. This count showed a marked increase in number of white blood cells. Out of a total increase of 6,700 white cells per c.mm. of blood, over 5,000 constituted the increase in polymorphonuclears. Now the fact that this phenomenon was delayed until after the administration of the drug, and then appeared so promptly, seemed to me to offer reason to surmise, at least, that there might be some relation between it and the pilocarpine.

*Curve Number II.*—This shows the operation to have been done at 9:40, and the first count to have been made at 9:48 o'clock. A second count at 10:23 showed an increase of about 3,000 polymorphonuclears per c.mm. This, of course, had no relation to the forthcoming dose of pilocarpine which was given at 10:26 o'clock. The third count, at 11:13, gave an increase of about 1,200 mononuclears, and a further increase of over 4,000 polymorphonuclears. This gradual increase in polymorphonuclear cells from the first would appear by itself to be the consequence of the operation inasmuch as it is not materially augmented by the 10 mg. of pilocarpine.

*Curve Number III.*—This shows the operation to have been done at 9:30, and, unfortunately for my purpose, no count was made until the end of an hour. However, the count at the end of the hour was too small to suggest any marked increase in leucocytes which might have been instituted by the operation. A half hour later 10 mg. of pilocarpine were given intravenously, and a count made at the end of another half hour showed an increase of 6,000 white cells per c.mm. of which the increase in polymorphonuclears constituted about 5,000. Another half hour gave a further increase of only 300 mononuclear cells per c.mm., but over 2,000 polymorphonuclears per c.mm.



CURVE IV.—A represents total white cells per cubic millimeter of blood; B, polynuclear cells per cubic millimeter of blood; C, mononuclear cells per cubicmillimeter of blood.



CURVE V.

These facts suggested to me some further experiments which I now describe briefly. First, to ascertain whether or not an operation, such as indicated above, would cause a leucocytosis so promptly and of such a degree, I exposed, after anæsthesia, an external jugular vein of a dog and inserted a cannula. A series of counts taken during the succeeding two hours showed no considerable change in total or in relative numbers of the various forms of white cells. The extreme variations were 1,200 polymorphonuclears and 250 mononuclears. This same observation was made on four other dogs subjected to other operations. There was certainly not such a leucocytosis from the operations as occurred in the animals after pilocarpine. This can be deduced from Curves VII and VIII.

The next step was to determine the effects of pilocarpine administration without any operation of consequence. The results are shown in Curves IV and V. In the first of these two cases, Curve IV, a count was made at the beginning of the anæsthetic, and another one fifteen minutes later, when anæsthesia had been established by means of chloroform followed by ether. These counts showed no considerable difference. Ten minutes later 15 mg. of pilocarpine hydrochloride were injected deep into the thigh muscles by means of an ordinary hypodermic syringe. This was followed by an immediate rise in the white blood-cell count. At the end of twenty minutes there had been an increase of 7,500 white cells per c.mm., of which the increase in polymorphonuclear cells was 6,000 per c.mm. In thirty minutes more the total increase had gone to 10,600 per c.mm. of which 8,528 were polymorphonuclear and 2,072 were mononuclear. Here we had a positive augmentation of polymorphonuclear leucocytes which can not at all be attributed to any operation, but certainly is a consequence of the pilocarpine. A determination of the percentages shows that the mononuclears increased from about 11 per cent. of the total to 15 per cent.

*Curve Number V.*—This represents practically the same experiment, in another dog, only prolonged somewhat, and with counts made more often. Within five minutes after the injection of pilocarpine there was a sharp increase in white cells, nearly 75 per cent. of this increase being in the polymorphonuclear type. Forty

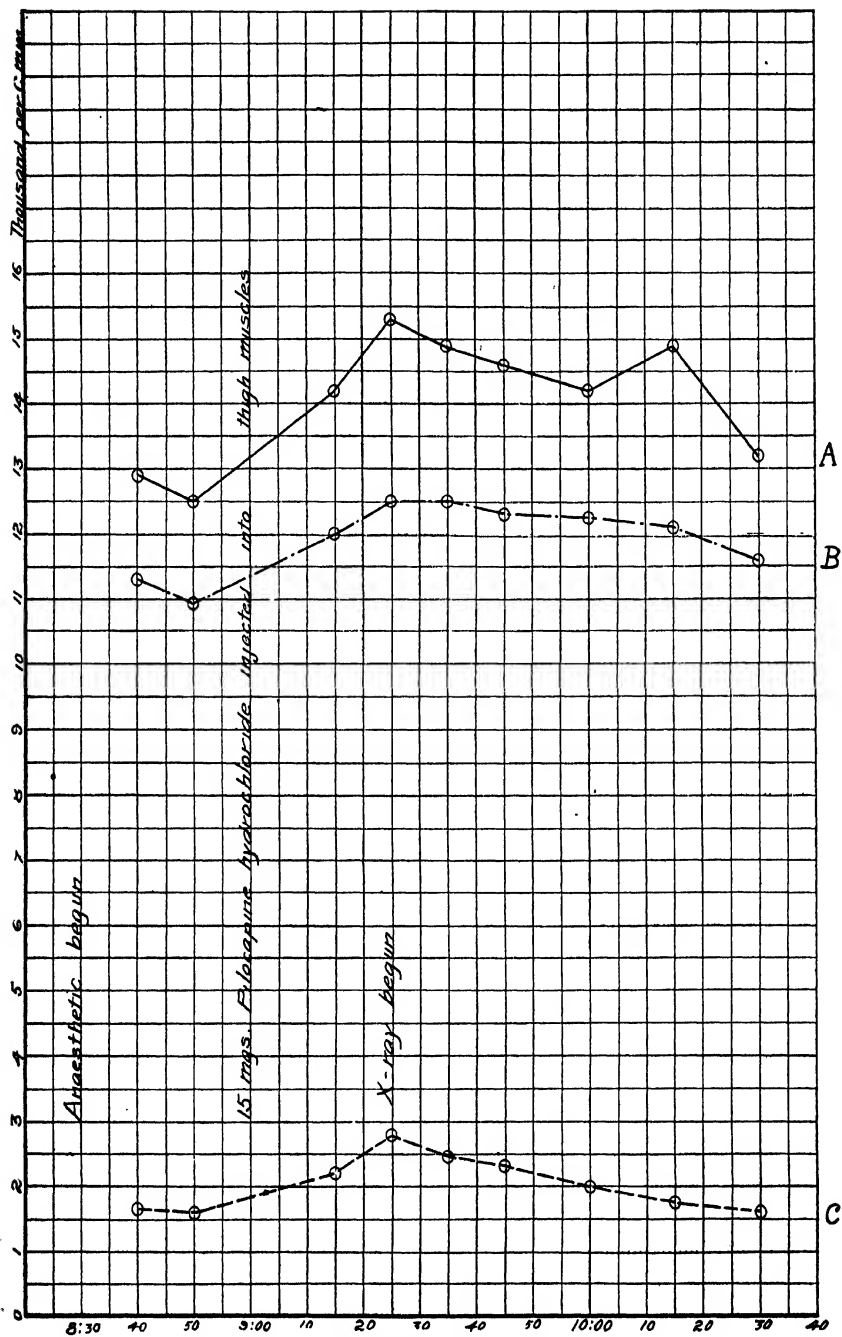
minutes after the drug was given the highest count was made. At this point the total increase consisted of 4,200 mononuclears and 4,000 polymorphonuclears per c.mm. During the succeeding thirty minutes there was a gradual decrease in the total count due almost entirely to a decrease in mononuclears. The final count showed the mononuclears to have gone 1,200 per c.mm. below the initial count, while the polymorphonuclears were still 3,200 per c.mm. above the count made at the time of beginning. This relative persistence of the polymorphonuclear increase should be given attention and will be considered further in connection with other curves showing the same feature. A determination of percentages in this case shows the mononuclears to have increased from 22 per cent. of the total to 32 per cent., and then to have gone down to 11 per cent. Such increases as are shown here, when considered on a basis of one hundred cells, would certainly attract one's attention to the lymphocytosis, as is the case in Harvey's report, but when the whole number of cells is taken into account the effect upon both types is noteworthy. The features prevailing in these experiments are also evident in the ones to be described presently, and so any conclusions which might be deduced here will be considered at the end of the next set of experiments.

The change in the lymphocyte count of the blood, produced by diverting from the blood stream the normal input by way of the thoracic duct, has been ascertained and established by several experimenters referred to above (Rous, Biedl and Decastello, Selnoff, Crescenzi, Parodi).

The following four experiments were calculated to furnish an opportunity to note the effects of Roentgen irradiation on the white blood-cells in the circulation, and on the cell content of the output from a thoracic duct fistula. The effect of the irradiation on the cell content of the circulating blood has received much attention heretofore, but, as far as I have been able to ascertain from the literature, no one has reported experiments to determine the effect on the cells in the content of the thoracic duct.

EXPERIMENT 1.—Female dog, 8 Kg., Curve VI.

The dog was anesthetized by chloroform followed by ether. Two blood counts were made ten and twenty minutes, respectively, later. These counts showed about 12,900 white cells per c.mm. of which 1,600 were mono-



CURVE VI.

nuclears. At the end of a half-hour 15 mg. pilocarpine hydrochloride were injected deep into the thigh muscles of the animal. Within twenty-five minutes, the total white cell count had gone up to 15,300 per c.mm. of which 2,800 were mononuclear. At this time the animal was brought into the field of Roentgen irradiation—medium tube, eight inches from the abdomen. There began a constant decline in the number of the white cells, particularly the mononuclear type. At the end of an hour the mononuclears had been restored to the original count while the total whites were still about a thousand higher than at the beginning. This, again, shows the persistence of the leucocytosis of polymorphonuclears even in spite of the Roentgen irradiation. In this instance the interruption of the pilocarpine effect by the X-ray is evident. The picture, however, is not nearly so striking as that shown by a test reported by Lefmann (9). Starting with a lymphocyte count of about 9,000 per c.mm. he increased it by injections of pilocarpine to 17,500 per c.mm. at which time he began the Roentgen irradiation and was able to reduce the count to 3,000 per c.mm. Then he suspended the X-rays and the number of lymphocytes rapidly went up to 23,000 per c.mm. He recorded the percentage which the lymphocytes bore to the whole number of white cells. By calculation on the basis of these percentages, it can be seen that there must have been a remarkable change in the number of polymorphonuclears as well. This is shown from the following: When the lymphocytes numbered 9,000 per c.mm. their percentage was 45; when they numbered 17,500 per c.mm. their percentage was 37; and when they numbered 23,000 per c.mm. their percentage was 38. Lefmann worked with rabbits in these cases and continued the experiments over several days, which matter will be considered later.

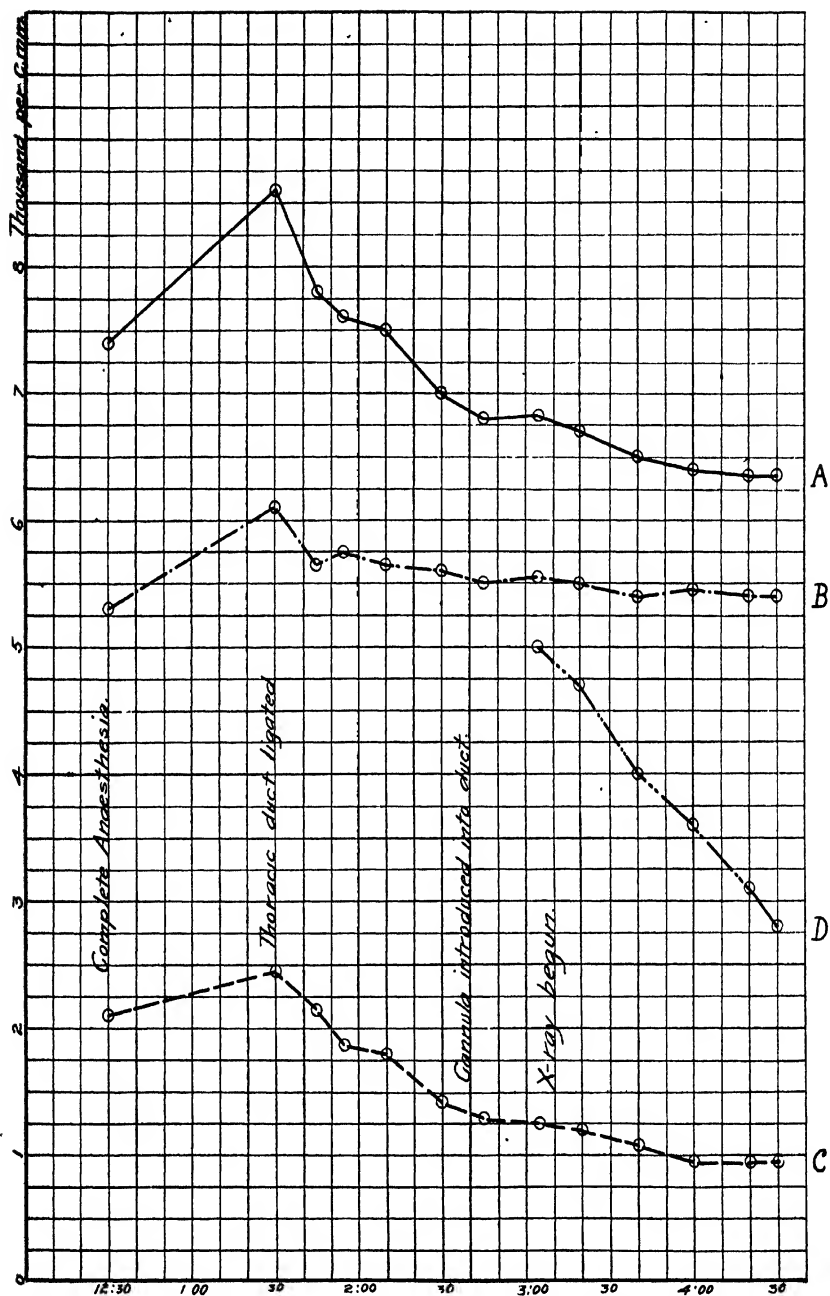
EXPERIMENT 2.—Male dog, 7Kg., Curve VII.

Anæsthesia was established by chloroform followed by ether. The initial count gave 7,400 white cells per c.mm. of blood, of which 2,100 were mononuclear. At the end of an hour the thoracic duct was ligated just before its entrance into the vein. The blood count at that time showed a rather great increase in white cells, apparently out of proportion to the operation and the time elapsed.

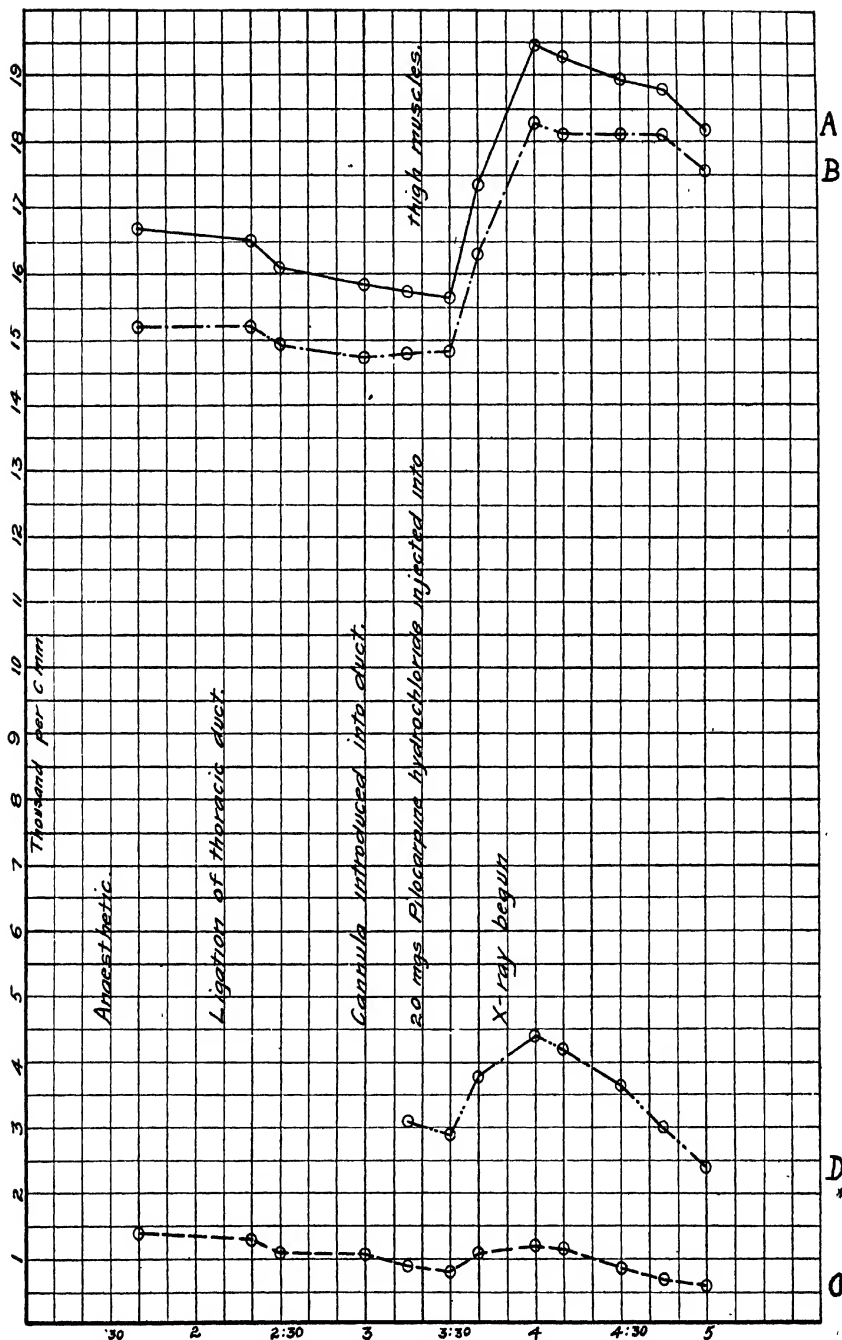
The next blood count, 15 minutes later, was near the original again, and prompts me to attribute this record of preliminary rise to error in making the count or in recording it. From then on the total whites decrease gradually, due in a great degree to a decrease in number of mononuclears. Later a cannula was introduced into the duct, and after the lymph had been allowed to flow twenty minutes, a count of the cells was made. This count gave 5,000 cells per c.mm. of lymph. Then the animal was placed in the field of Roentgen irradiation—medium tube, 8 inches from abdomen—and successive counts made of the cells in the blood, and of the cells in the outflow from the duct. In the blood the white cell number decreased generally, with some fluctuations in both types. In the duct output there occurred a definite and marked decrease in cell-content, so that at the end of an hour and a half the number of mononuclear cells had fallen from 5,000 per c.mm. to 2,800 per c.mm.

EXPERIMENT 3.—Male dog, 14 Kg., Curve VIII.

Anæsthesia was begun at 1:30 o'clock, chloroform followed by ether being used. At 1:40 the blood count showed total white cells to be 16,700 per c.mm.



CURVE VII.—D represents output of mononuclear cells per cubic millimeter from thoracic duct.



CURVE VIII.

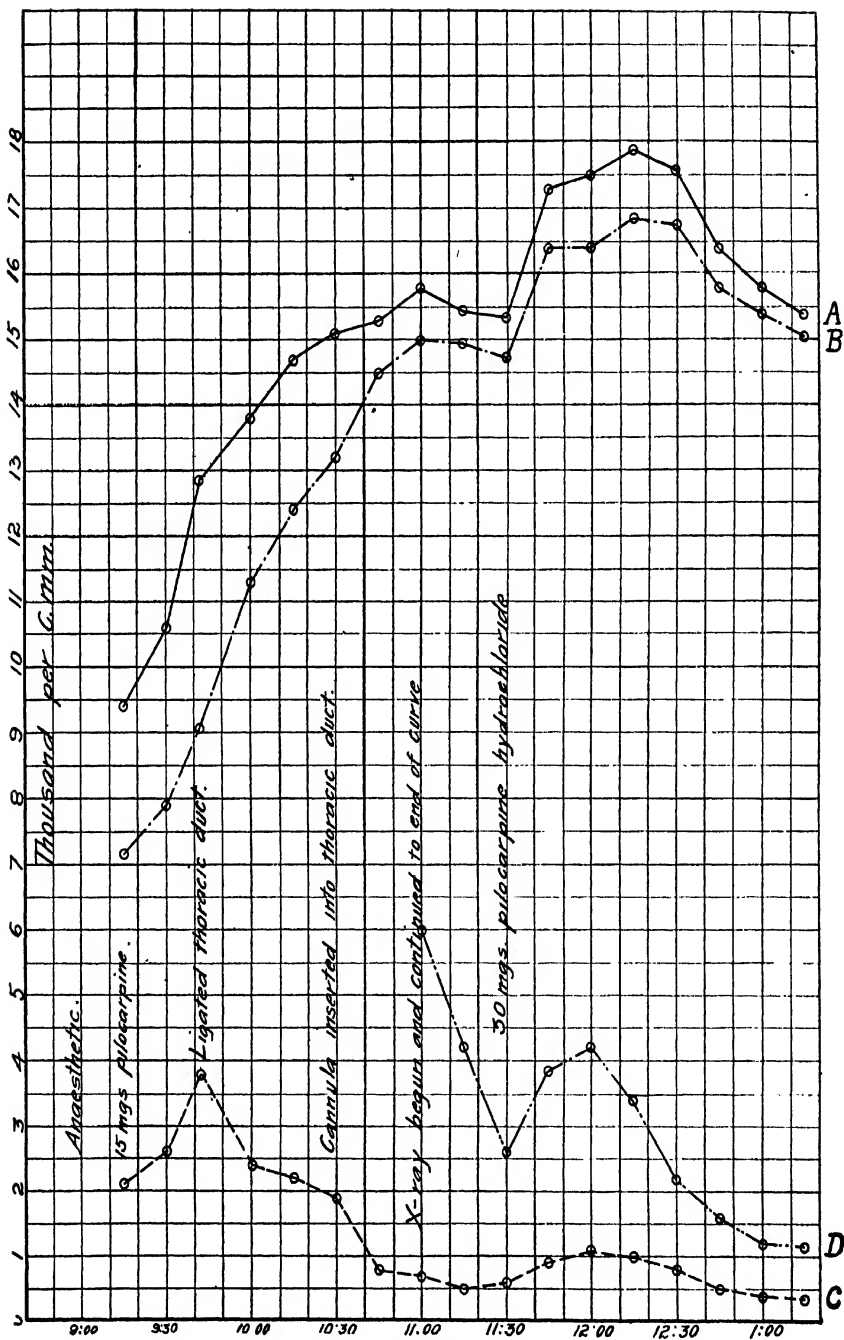
of which 1,400 were mononuclear. At 2:10 the thoracic duct was ligated, and at 3:00 a cannula had been inserted and the lymph was allowed to flow. At 3:15 o'clock the blood count showed a decrease in white cells, characterized by decrease in lymphocytes. At this time the output from the thoracic duct fistula contained 3,100 white cells. Immediately 20 mg. of pilocarpine hydrochloride were injected into the thigh muscles. The next counts at 3:30 gave still a decrease in white cells in the blood and in the duct output. At 3:40 there was an evident increase established, and the animal was exposed to Roentgen irradiation—medium tube, 8 inches from abdomen—at 3:45. The count at 4:00 o'clock gave still an evident increase of white cells in both blood and lymph, but each successive count, at intervals of about fifteen minutes, showed marked decrease in mononuclear cells in the circulation and in the lymph. At 5:00 o'clock the polymorphonuclear type remained 2,500 above the initial count, while the mononuclears were below any previous count.

This curve reaffirms the previous observations viz., (1) the increase in polymorphonuclears induced by the drug, while not so great in percentage, is remarkable in consideration of the great number of cells; (2) the persistence of the high count of polymorphonuclear cells in spite of the Roentgen rays; (3) the interruption of the lymphocytosis by Roentgen irradiation; (4) the relatively slight effect of the X-rays on the polymorphonuclear cells as compared with the effect on the mononuclear cells.

EXPERIMENT 4.—Male dog, 10 Kg., Curve IX.

Anæsthesia by chloroform followed by ether was established between 9:00 and 9:15 o'clock. Then 15 mg. of pilocarpine hydrochloride were injected deep into the thigh muscles. The white cells in the blood immediately increased. After ligation of the thoracic duct at 9:45 the mononuclear count in the blood fell, with a still increasing polymorphonuclear count. At 10:30 a cannula was inserted into the duct and the lymph allowed to flow. At 11:00 o'clock the lymph contained 6,000 mononuclear cells per c.mm., and at that time the Roentgen irradiation—medium tube, 8 inches from abdomen—was begun and continued to the end of the experiment. There followed a rapid fall in the white-cell number in the lymph, a small decrease in mononuclear cells in the circulation, and a relatively slight decrease in polymorphonuclears. I do not attribute this great fall in cell number in the lymph to be due entirely to the action of the X-rays, but in part as a consequence of the congested condition of the duct due to ligation. At 11:30, thirty milligrams of pilocarpine hydrochloride were injected. This heavy dose caused a second rise in cell counts, which, however, was soon interrupted by the continuing irradiation especially as far as concerns the mononuclear cells. At the close of the process the polymorphonuclear cells were nearly 8,000 per c.mm. above the initial count, while the mononuclear elements were very much diminished.

This curve gives reason for especial consideration, although it does not offer any features not previously shown. In the first place the action of the drug is plainly on both types of cells, with the percentage in favor of the mononuclears. Then the effect of shutting off from the circulation the usual input by way of the duct is



CURVE IX.

very striking, when we consider what the further effect of the drug would have been. Accompanying this we have the continued rise in polymorphonuclear cells, which is but slightly affected by the irradiation. Then the second and relatively large dose of pilocarpine, acting in opposition to the irradiation causes a secondary rise in all elements, which is of short duration and is succeeded by gradual and definite decline especially in the lymph.

There are some points which render it impracticable to make definite and unlimited conclusions from the foregoing sets of experiments. The variations in the thoracic duct and its tributaries render it impossible to turn completely from the circulation the great bulk of the lymph. By injecting the duct from the receptaculum upward with a colored solution various branches and irregularities become evident which could not be ascertained and taken into account in the process of establishing a thoracic duct fistula. This point might affect materially the consideration of how and where the Roentgen rays effect a diminution in lymphocytes. This will be taken up in connection with my conclusions on this point.

Another factor of consequence in calculating on the count per cubic millimeter of lymph is the variation in fluid constituent. The small variations in cell count may be only relative and not absolute. This will not affect general conclusions, but might be responsible for some of the details of a conclusion. Rous made definite note of rate of flow and absolute count of cells in the lymph and showed that practically all of the lymphocytes in the general circulation might have been supplied by this source or route.

In making the total counts and also the smears for differential counts, I made use of single drops of lymph as they appeared at the end of the cannulæ, rather than making counts from several cubic centimeters, because I considered that the latter method would not give the changes in as definite relation to time and degree on account of the time necessary to collect the bulk. The matter of struggle on the part of the animal is also of consequence (Rous (1)). In these cases the anæsthesia was maintained so completely as to preclude any struggle.

The effects of the operation must be considered. Whether or

not an operation necessary to establish a thoracic duct fistula will immediately and definitely increase the white blood-cells seems to be a question. In these cases I found no satisfactory grounds for the conclusion that such is the result.

The apparently normal variations in the relative number of the various types of cells in the dog's blood, and especially the number of cells which cannot be said positively to belong to one or another type, enter into consideration materially at times.

In making the differential counts I have discarded the cells which I could not consider with a reasonable degree of certainty to belong to a certain class.

The stability of the blood of the animal used should enter into consideration. The experiments reported as done on the rabbit should be interpreted with this in view. This is especially true when the duration of the experiments is a matter of several days, and the intervals between counts are several hours. Surely it is uncertain to attribute the great changes, even, which might then appear as consequences of the drug used or of the Roentgen rays. For that reason I have done all of these experiments on dogs and used short periods of time with heavier doses of drug and irradiation.

With these possible variations in mind, I feel justified in stating some definite conclusions from the foregoing sets of experiments.

I. *Polymorphonuclear Cells*.—1. Relatively slight operations on dogs do not cause any great degree of leucocytosis within the limits of time consumed in these experiments. The substantiation of this point has been explained under the curves calculated to give the basis for the conclusion.

2. Injection of pilocarpine hydrochloride into the muscle of a dog brings about a decided increase of polymorphonuclear cells in the circulation. This is shown in every case under consideration. The increase in percentage is usually about the same as that of the mononuclear cells, but is much greater in absolute counts.

3. The polymorphonuclear leucocytosis is more persistent than the mononuclear change. This is shown in Curve V, in which case the X-ray was not used, and in Curves VIII and IX in which cases irradiation was applied.

4. The polymorphonuclear cells are but relatively slightly re-

duced in number by the action of the Roentgen rays. This is shown in Curves VI, VII, VIII, and IX.

II. *Mononuclear Cells*.—I. In the blood—(a) The increase in number of mononuclear cells in the blood following injection of pilocarpine hydrochloride is definite. (b) This increase is interrupted by the action of the X-rays and is more susceptible to their action than that of the polymorphonuclear cells. (c) The number is materially diminished by diverting from the blood stream the normal input by way of the thoracic duct.

2. In the output from the thoracic duct—(a) The number in this output is great enough, considering rate of flow and bulk of blood and lymph, to account for practically all the lymphocytes in the blood and so gives reason for the conclusion that this is the most important route by which they reach the blood. (b) The injection of pilocarpine hydrochloride causes a marked increase in the number entering the blood by this route (Curves VIII and IX). (c) This increase is interrupted by the action of Roentgen irradiation, and the number can be reduced to below the initial count (Curves VI, VII, VIII, IX).

III. *Roentgen Irradiation*.—I. On the cells in the circulating blood there is a noticeable effect. This is shown in a diminution of all forms of white cells. In each case in which the Roentgen rays were used there is shown a much greater decrease of mononuclears than of polymorphonuclears. This is perhaps due to the fact that the parent cells of the polymorphonuclear cells were not so accessible to the action of the X-rays on account of the bone tissue enclosing them, and because the long bones of the legs were practically out of the field, while the spleen and the great numbers of lymph nodes were directly in the course of the rays and not so well protected by bone.

2. The effect of the irradiation is most plainly evident on the cell content of the lymph from the thoracic duct. In each curve this is shown. From this feature of the results it is evident that the great effect of Roentgen irradiation on the mononuclear cells in the circulating blood can be explained to a great degree, at least, by its action on these cells before they reach the circulation through the thoracic duct. The fact that not all of the lymphocytes can be diverted from the blood may be made use of to explain the changes

which apparently go on in the blood under X-ray treatment after ligation of the duct. Together with this must be considered the fact that a greater body-area than the region giving supply to the thoracic duct is being irradiated, and such changes as are produced in the blood, after ligation of the duct, may be the effects of the irradiation on these accessory regions, such as the bone marrow.

It would seem then that the theory of the production of a leucotoxin in the blood, as put forward by Linser and Helber (12) and Capps and Smith (13) is not necessary to explain the destructive action of Roentgen rays upon the white cells. As has been pointed out by Rous, there is much difficulty in classifying the cells from the thoracic duct lymph. The irregularities in the cells resulting from making the smears cause this difficulty. So in considering the effect of the pilocarpine or of the irradiation on the individual cell of the lymph, one should scarcely consider the form of the cell. From the staining reaction I could not say that the number of degenerated forms was increased after the irradiation, and should rather consider that such degenerated cells as resulted from the irradiation were retained in the blood-cell forming organs and not given into the circulation. These organs in these animals were not studied to ascertain their condition in this particular.

In reporting this investigation I am under especial obligations to Professor Warthin, who has given the work much of his attention. I also wish to thank Mr. Willey and Mr. Hill, of the Roentgenology Department, for much assistance and the use of their laboratory.

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# A CHEMICAL STUDY OF THE BRAIN IN HEALTHY AND DISEASED CONDITIONS, WITH ESPECIAL REFERENCE TO DEMENTIA PRÆCOX.

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## I.—INTRODUCTION.

At the suggestion of Dr. F. W. Mott, F.R.S., the methods for the chemical study of the brain outlined in a previous paper (1) by one of us (W. K.) have been extended to the study of certain mental disorders. In the course of the work the above methods have been frequently revised and elaborated, and we take this opportunity of republishing them in their amended form.

Attempts have been made by various observers to study the chemistry of mental disorders by analysis of the various bodily fluids and excretions, such as the blood, cerebrospinal fluid and urine. Results obtained by this method are difficult of interpretation and are liable to lead to erroneous conclusions. With regard to the examination of the urine, Folin and Schaffer (2), using carefully elaborated analytical methods, have completed an exhaustive work, and have come to the conclusion that there is little information to be obtained by attacking the problem solely through this source, and, further, that the large number of observations on record based on analysis of the urine and regarding the relation of an abnormal metabolism of the body to mental derangement, are of little value. The more recent observation of Pighini (*Vide* "Archives of Neurology and Psychiatry," Vol. IV., p. 220), that there is an increase of neutral sulphur in the urine in dementia præcox can be explained as being due to a general decrease in the oxidations and can be produced experimentally, as Richards and Wallace (3) have shown, by cyanide poisoning. This result, however, is of interest in view of the change in the neutral sulphur content of the brain (*Vide* "Archives of Neurology and Psychiatry," Vol. IV., p. 209) in this form of mental disorder.

The cerebrospinal fluid bears a more direct relationship to the nervous system and offers good opportunities for the chemical study of the products of nervous metabolism, but the amount of fluid which can be safely withdrawn by lumbar puncture during life is not sufficient to allow of accurate quantitative estimations. Qualitative tests, however, have shown in certain morbid conditions the presence of various products of degeneration, *e.g.*, choline (Mott and Halliburton (4)), and in general paralysis especially, a disease in which the fluid is in great excess, the protein and lipid constituents are found to be increased in amount. In view of the significance which has been attached to the Wassermann Plaut reaction in cases of general paralysis and other parasymphilitic affections, the further study of the protein and lipid constituents in the serum and cerebrospinal fluid of these cases is of great importance in deciding the chemical nature of the substance causing the complement deviation.

*Examination of the nerve tissue*—(a) *Micro-chemical*.—By far the largest number of investigations of the nervous system itself have been concerned with such histological methods of staining as devised by Weigert, Nissl, Golgi, Ramon y Cajal, Held, and others. These investigations have shed light on the anatomical structure of the brain, but on account of our great lack of knowledge of the chemical constituents of the nervous system which are involved in these reactions, comparatively few have been regarded in the light of micro-chemical reactions. Gustav Mann (5), in his excellent text-book of physiological histology has stated practically all that can be said on the subject. Micro-chemical methods may decide points of anatomical distribution of constituents in a qualitative manner and correlated with quantitative macro-chemical observations lead to important conclusions. Thus in two cases of amaurotic dementia Mott (6) was able to associate the disappearance of the Nissl substance in the neurons with a decrease in nucleo-proteid, and an increase of the glia fibrils with an increase of simple proteid, the brain in each case being chemically examined by one of us (S. A. M.).

*Examination of the nervous system*—(b) *Macro-chemical*.—The study of the quantitative variations in the composition of the brain under normal and pathological conditions has so far received but little attention. In fact, this may be said of any tissue of the body on account of our lack of knowledge of the substances to be estimated. Our present knowledge permits us only to refer to groups of substances which in the nervous system may be arranged under the following general headings:—

1. Lipoids.—Phosphatids, cerebrins, cholesterin, and a sulphur compound.

2. Extractives.—Organic water soluble compounds not colloidal in nature, *e.g.*, kreatin, taurin, hypoxanthin, etc.

3. Inorganic constituents.—Ash.

4. Proteins.—Nucleoproteins, globulins, neurokeratin.

A more detailed account of the chemical constituents isolated from the brain was given in the 1904 paper, and for the sake of completeness it is here brought up to date.

#### CHEMICAL CONSTITUENTS OF BRAIN TISSUE.

1. WATER.— $H_2O$ , present in largest amount.

2. SIMPLE AND COMPOUND PROTEINS (C, H, O, N, S, P).

*Globulin* coagulating at  $47^{\circ}$ – $50^{\circ}$  C. (Halliburton (7)).

*Globulin* coagulating at  $70^{\circ}$  C. (Halliburton (7)).

*Neurostromin* (Schkarin (8)).—Extracted by sodium hydrate, present only in small amount.

material by Thudichum (11, p. 178) and Thierfelder (16), on sheep's brains by Koch (15), and on horses' brains by Bethe (17, p. 78), and more recently confirmed by Rosenheim. As there is very little difference in the cerebrins derived from different species it will simplify matters to compare them regardless of their source.

*Phrenosin*,  $C_{41}H_{79}NO_8$ . The substance isolated by Thudichum (11, p. 184) may be said to be identical or isomeric with that isolated by Thierfelder and by Koch, as will be seen from a comparison of the analyses:—

	THUDICHUM.	THIERFELDER	KOCH.
Carbon ...	69.00	69.16	68.73
Hydrogen ..	11.08	11.54	11.83
Nitrogen .	1.96	1.76	1.64

Phrenosin spits off galactose on heating with dilute mineral acids.

*Kerasin*,  $C_{44}H_{88}NO_8$  (Thudichum (11), p. 218), probably identical with Bethe's (17, p. 184) *amidocerebrin acid glycosid*,  $C_{44}H_{81}NO_8$ . Both are undoubtedly homologues of phrenosin, and in the same way split off galactose.

*Phrenin*, isolated by Bethe (17, p. 184), was obtained by Koch as a decomposition product from cerebrin after boiling with dilute hydrochloric acid. The analyses agree fairly well.

	BETHE.	KOCH.
Carbon ..	71.90	71.60
Hydrogen ..	11.95	12.14
Nitrogen ...	1.5	1.89

This substance does not split off a reducing sugar as do phrenosin and kerasin.

*Cerebrin acids* so far only isolated by Thudichum (11, p. 221).

*Cerebrin acid*,  $C_{49}H_{99}NO_{11}$ , and *sphaerocerebrin*,  $C_{58}H_{123}NO_{17}$ , are characterised by forming lead salts insoluble in hot alcohol. This distinguishes them from kerasin and phrenosin, which do not combine with lead. Cerebrin acid, according to Thudichum, splits off galactose. A comparison of the percentage of carbon found by Thudichum makes it extremely probable that these substances are intermediary oxidation products of phrenosin and kerasin, as indicated by the following figures:—

	PHRENOSIN.	CEREBRIN ACID.	SPHAEROCEREBRIN
Carbon ..	69.0	67.00	62.75
Hydrogen ...	11.08	11.36	11.08
Nitrogen ...	1.96	1.59	1.23

No other substances have been isolated which may be said to belong to the group of the cerebrins. The cerebrinphosphoric acid of Bethe is

only an impure mixture which undoubtedly contains sulphur, for which he neglected to test. None of the members of this group can be said to be pure until they are free from sulphur and phosphorus, both of which elements cling to them most tenaciously. All the cerebrins when pure are insoluble in ether and soluble in hot alcohol. The cerebrin acids are more soluble in glacial acetic acid than phrenosin.

7. CHOLESTERIN (C, O, H),  $C_{27}H_{45}OH$ , occurs in the brain as free cholesterin and not in the form of esters. Soluble in hot alcohol and cold ether. Bünz (18). Tebb (19).

8. SULPHUR COMPOUNDS (C, H, O, N, P, S).—Thudichum (11, p. 224), Koch (13).

9. AMIDOFATS (C, H, N, O).

*Krinosin*,  $C_{38}H_{79}NO_5$ .

*Bregenin*,  $C_{40}H_{81}NO_5$ .

These substances have so far been only isolated by Thudichum (11, p. 227). They are distinguished from fats by their insolubility in ether. The quantitative determinations indicate that they may be present in small amount only. They most probably represent *post-mortem* decomposition products.

10. MONOPHOSPHATIDS (C, H, O, P).

*Lipophosphoric acid*, *butophosphoric acid*.—Isolated by Thudichum (11, p. 177), but not analysed completely. Contain about 4 per cent. of phosphorus, and are free from nitrogen. May be present in slight amount in white matter, as not quite all the phosphorus is accounted for. Most probably, however, they are either *post-mortem* decomposition products or the result of chemical manipulation.

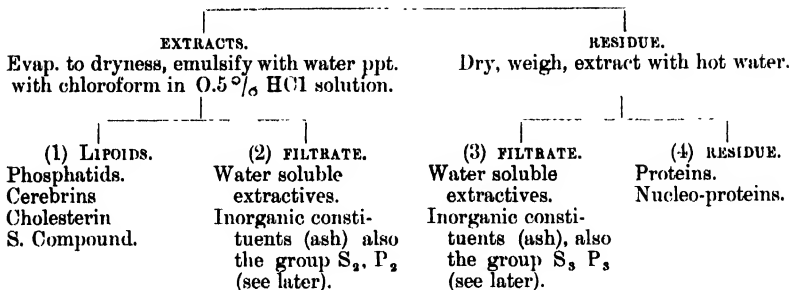
This completes the list of substances isolated or supposed to have been isolated from brain tissues. Free fats and fatty acids have never been found to be present in normal brain tissue. Bethe (17, p. 86) mentions stearic acid but adds a question mark, which is a wise provision, as he has been rather unfortunate in describing decomposition products as primary constituents (phrenin, *see above*). Protagon we have no intention in resurrecting, in spite of fears to the contrary (20), as the work of Thierfelder (16), Gies (21), and Rosenheim and Tebb (22) has settled its fate. For further discussion on this subject, *see also* Cramer (23, 24).

*Principles of estimation of constituents*.—These constituents are separated into the four general groups mentioned above (p. 3) by solvents according to the following outline: Alcohol has proved to be the most satisfactory solvent, and, although it is desirable in a quantitative study of this kind to rely on methods of separation by solvents as little as possible, observations which are recorded later

(p. 15), indicate that the separation of lipoids from the proteins is as good as can be found at present.

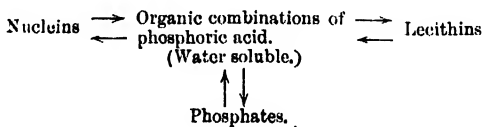
#### MOIST TISSUE.

Add alcohol and extract alternately with alcohol and ether.



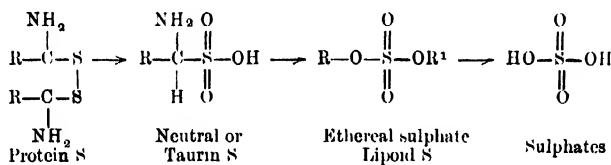
Besides studying the variations of the constituents as cerebrin, protein, which may be looked upon as the *food* supply of the brain, in normal and pathological cases, the variations of the distribution of the elements phosphorus and sulphur among the various groups were also investigated. The selection of these two elements was based on the following considerations.

*Phosphorus* occurs in the body exclusively as combinations of phosphoric acid, an oxidised derivative. Its mechanism of absorption and intermediary metabolism is not yet fully understood, but it is quite certain that it enters the system either as a phosphate or some simple organic derivative and leaves in the form of an acid phosphate. It is the radicle which seems to play the rôle in the building up of the most complex constituents of the cell, the nucleins and phosphatids. The variation in its distribution between these and the water soluble extractives should therefore give an indication of the amount of destruction of these important cell constituents. The following outline will serve to make this clear:—



A relative increase of the water soluble phosphoric acid derivatives may be interpreted as being due to an excessive breakdown of nucleins and lecithins, or may occur during a condition of rapid growth where the food materials are supplied in abundance (25). During a condition of starvation, where, however, the reparative reaction is still going on, phosphates should be decreased (26).

*Sulphur* occurs in the body in various stages of oxidation. As —SH or cystin sulphur in proteins, as sulphonate or  $\text{R}-\overset{\text{O}}{\underset{\text{O}}{\underset{\text{O}}{\text{N}}}}\cdot\text{OH}$  or taurin like sulphur, and as ethereal and inorganic sulphates. Sulphur probably enters the organism largely as unoxidised or cystin sulphur and leaves to the extent of 95 per cent. in an oxidised form as inorganic sulphates. It differs, therefore, from phosphorus in undergoing a change in its state of oxidation as a result of the intermediary metabolism. It seemed possible, therefore, to utilise the variation in the different stages of oxidation of sulphur for measuring the extent to which oxidising reactions are going on in the tissues, especially as the work of Heffner (27) shows that the affinity of the sulphur group for oxygen plays a very important rôle in the organism. The relations are made plain by the following scheme:—



## II.—EXPERIMENTAL PART.

*Methods.*—Before entering into the details of the methods adopted, we consider that it will save the reader much confusion if we state in full the symbols by which we have designated the various sulphur and phosphorus fractions. Incidentally, we put this forward as a system of labelling, for it must be remembered that the examination of one brain lasts nearly one month, also the various fractions are constantly being transferred from one vessel to another, and when a few brains are being examined at the same time, a definite system of labelling must be adhered to.

The general scheme of separation then leads to four fractions:—

1. Alcohol soluble, insoluble in acid chloroform water (lipoids).
2. Alcohol soluble, soluble in acid chloroform water (extractives).
3. Alcohol insoluble, water soluble (extractives).
4. Alcohol insoluble, water insoluble (proteins).

It is in these various fractions that it is proposed to study the distri-

butions of the elements sulphur and phosphorus. These various fractions may then be designated :—

S<sub>1</sub>. Alcohol soluble, acid chloroform water insoluble, lipid sulphur.

S<sub>2</sub>. Alcohol soluble, acid chloroform water soluble, extractive sulphur.

S<sub>3</sub>. Alcohol insoluble, water soluble, extractive sulphur.

S<sub>4</sub>. Alcohol insoluble, water insoluble, protein sulphur.

S<sub>1</sub><sup>2</sup> and S<sub>1</sub><sup>3</sup> represent inorganic sulphates, derived from these fractions by direct treatment with barium chloride.

The same applies to the phosphorus fractions.

P<sub>1</sub>. Alcohol soluble, acid chloroform water insoluble, lipid phosphorus.

P<sub>2</sub>. Alcohol soluble, acid chloroform water soluble, extractive phosphorus.

P<sub>3</sub>. Alcohol insoluble, water soluble, extractive phosphorus.

P<sub>4</sub>. Alcohol insoluble, water insoluble, protein phosphorus.

P<sub>1</sub><sup>2</sup> and P<sub>1</sub><sup>3</sup> represent inorganic phosphates derived from these fractions by direct precipitation with magnesia mixture.

P<sub>1</sub><sup>L</sup> and P<sub>1</sub><sup>K</sup> represent lecithin and kephalin phosphorus as separated by the lead kephalin salt.

Such a system of notation is of great value in a large number of analyses. By adding the case number it is possible to tell after several months just how the material has been handled. Thus 23 P<sub>1</sub><sup>L</sup> × 5 refers to sample 23 and means the lecithin fraction of the total lipid phosphorus multiplied by 5 on account of the fact that an aliquot part was taken. 70 S<sub>1</sub><sup>2</sup> means that in sample 70 the hot-water extract of the alcohol insoluble residue was precipitated direct with barium chloride in hydrochloric acid solution.

#### COLLECTION AND PRESERVATION OF MATERIAL.

The previous method of collecting the material by separation of the grey and white matter was only followed in a few cases, which will be given later. It soon became evident that, especially in the case of the grey matter, too much time would be required to collect the amount of material necessary to ensure accurate analyses in some of the sulphur and phosphorus fractions. As changes in the metabolism of the nervous system such as we are here seeking to investigate are more apt to affect the brain as a whole, it seemed advisable to take larger samples, and one half of the brain was used for chemical work and the other retained for histological examination.

The half of the brain intended for chemical work can be used either as a whole or for the separation of the cortex and corpus callosum. The membranes are removed and any blood washed away, and the brain

allowed to drain. This procedure vitiates any absolute estimation of the amount of moisture, but as this figure was never used except to refer the constituents to the per cent. of total solids, there is no error involved.

(a) *Collection of white matter from corpus callosum and adjacent centrum ovale.*—As much as possible of the corpus callosum and centrum ovale is dissected and all adhering particles of grey matter removed. It is then finely minced with scalpels and a two-gramme sample taken for the moisture determination. The remainder (50 grammes) is transferred to a 250 c.c. bottle and weighed. Absolute alcohol is added to nearly fill the bottle and the whole is well shaken. The following day the bottle is again shaken, heated to 75° C. by immersion in water at that temperature, and set aside for future analysis.

(b) *Collection of grey matter from cortex.*—The frontal and motor regions are utilised and the grey matter is trimmed off with a sharp scalpel without any adhering white matter. About 50 grammes of cortex is collected in this manner, minced, and mixed as well as possible, two grammes taken for the water estimation, and the remainder weighed and preserved the same as the corpus callosum. This mode of collecting the grey matter takes a considerable time, and requires much care and patience.

(c) *Collection of mixture of grey and white matter from the whole hemisphere.*—The whole of one hemisphere is passed through a mincing machine with a fairly fine wire mesh ( $\frac{1}{8}$ -inch), well mixed and again passed through the mincing machine, and *three* one-hundred-gramme samples taken. A sample this size ensures a sufficiently uniform mixture of the white and grey matter (*vide* p. 27), and gives amounts of barium sulphate and magnesium pyrophosphate in the various sulphur and phosphorus fractions large enough to give accurate weighings, and does not involve the extraction and destruction of inconveniently large amounts of organic material. The *three* 100-gramme samples are preserved in at least 400 c.c. of absolute alcohol each; allowing for the amount of moisture in the sample, this ensures a concentration of about 83 per cent. alcohol in the preserving fluid.

As some of the samples were analysed at different periods of time after the collection of the material, it seems of interest to compare them from the point of view of this method of preservation:—

Time between collection and analysis of sample	3 hours	2 days	2 weeks	3 months
Sulphur in alcohol soluble fraction	42.7	38.4	36.9	35.9
Sulphur in alcohol insoluble fraction	57.3	61.7	63.1	64.1
Phosphorus in alcohol soluble fraction	86.3	87.5	84.7	83.8
Phosphorus in alcohol insoluble fraction	13.8	12.5	15.3	16.3

The changes to be observed in the sulphur are evidently not due to lack of preservation, but indicate rather an incomplete coagulation of the proteins. After two or three weeks this coagulation seems to be complete, and the results then are more uniform. It is well, however, on the day after collecting the sample to thoroughly shake up the mixture so as to ensure complete penetration of the alcohol into the tissue, and to heat it up to a temperature just below the boiling point of alcohol by placing the bottle containing the sample in a water bath at 75° C.

One other factor needs to be considered in the collection of the material, *i.e.*, the amount of variation introduced by the time the material is kept after death before preservation in alcohol. It is quite impossible to avoid the immediate *post-mortem* changes which occur at the moment of cell death or possibly just before; but as the hospital and asylum brains were handled in much the same way this variation becomes constant. The differences between a brain collected one hour or thirty hours after death, however, need to be considered. As the differences are apt to take the form of autolysis or breaking up of the complex colloidal molecules into simpler ones they would influence the relation of the different fractions. The best means of measuring these would be by a study of the changes in the nitrogen, such as have been done frequently in studies of autolysis. As the nitrogen, however, has not been considered in these analyses, a comparison of the extractive or water soluble phosphorus will serve the purpose:—

Time of collecting after death.	Water soluble extractive P.	Lipoid and Protein P.
1 hour .. .. .	26.9	73.0
4 hours .. .. .	24.7	75.2
17 hours .. .. .	24.7	75.3
19 hours .. .. .	23.2	76.7
30 hours (cold chamber)	25.1	75.0

The results, if anything, show a change contrary to what might be expected if autolysis were proceeding, and, in view of the fact that these analyses were made on different samples, the variations are of little significance. The results are in harmony with other observations which indicate a very slow rate of autolysis in the brain. Nevertheless, the material should be collected within 24 hours after death if possible.

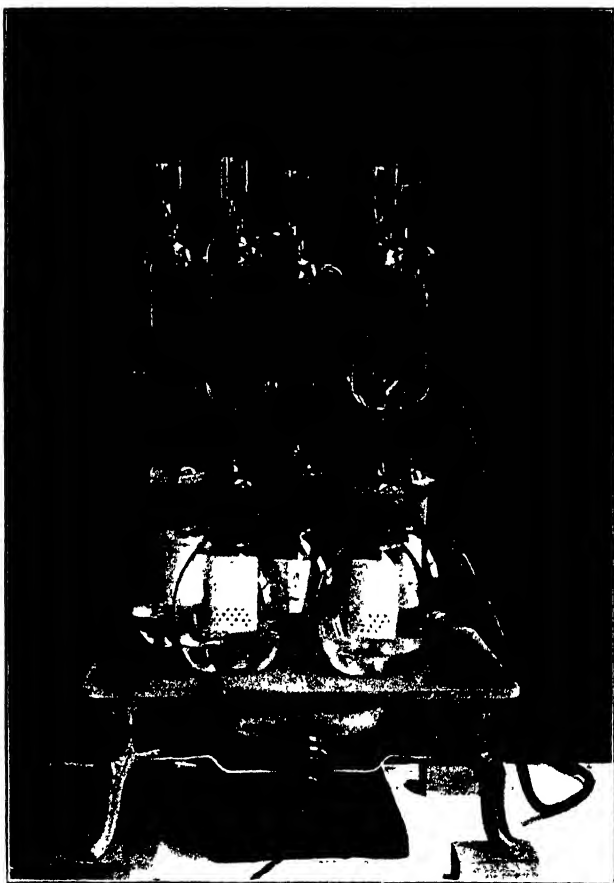
#### METHOD OF EXTRACTION—OUTLINE OF METHOD.

In the plan previously outlined (p. 8) for the separation of the different groups of constituents the following fractions are first obtained:—

- 1 and 2. Fraction soluble in alcohol (85-95 per cent.).
3. Fraction insoluble in alcohol, soluble in hot water.
4. Fraction insoluble in alcohol and hot water.



PLATE I.



Although ether is used in the extraction following the first alcohol, it does not remove any considerable amount of material and need not be considered in the above scheme. The apparatus used for the extractions is a modification of the old form of Wiley extraction apparatus, and was designed for us, according to our suggestions, by Gallenkamp and Co., London. The advantage of this form over the Soxhlet, especially for work with nerve tissues, consists in the fact that the extraction takes place at the boiling point of the solvent.

The apparatus (Fig. I.) consists of a wide mouth  $\text{CO}_2$  flask of 300 c.c. capacity into which it is fitted, by means of a ground glass connection, a small double surface condenser. On the lower end of the condenser are

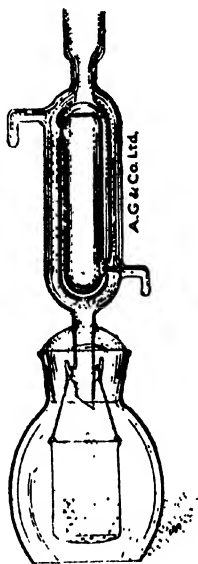


FIG. I

fused two glass hooks, from which is suspended in a thin platinum wire sling a 40 c.c. perforated cup. Each condenser is fitted with at least three interchangeable flasks. If dry heat is used for the extraction (cf. Electric Plate, Plate I.) the above form of condenser answers admirably, but should a water bath be used, the escaping steam will condense on the external surface of the condenser and, creeping into the ground glass connection, will possibly cause it to stick. With a water bath, a Hopkin's condenser (Fig. II.)—in which the cooling is accomplished from the inside—prevents this source of annoyance. Electric hot plates have the disadvantage that they are heated irregularly, but we have found

them, *used carefully*, to effect a great saving in time, and also to eliminate all risk of fire. Plate I. shows a number of extractions proceeding at the same time; these, when once started, require but little supervision, and can be left running during the night for the final extractions.

For the extraction the material is transferred to the perforated cups and first extracted for 3-4 hours with 95 per cent. alcohol. As this removes about nine-tenths of the alcohol soluble portion, it is better to discontinue heating on account of the danger of decomposing some of the compounds now in the alcohol solution. The alcohol extraction is

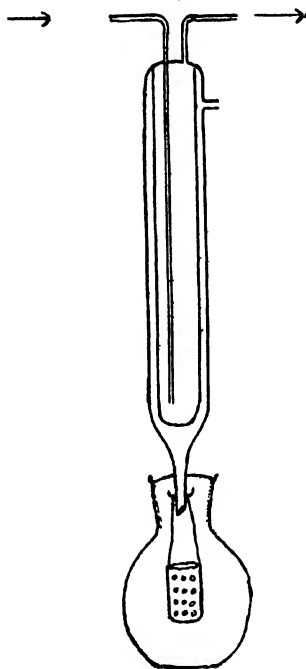


Fig II.

followed with an ether extraction, and this, although it does not remove much material, has the advantage of rendering the nerve tissue extremely brittle, so that it can be finely powdered, which procedure greatly aids the completeness of the later extraction. After powdering, the material is mixed with water and heated; absolute alcohol is then added to bring the concentration to at least 85 per cent. This procedure ensures the more complete extraction of those extractives, which are only slowly soluble in 95 per cent. alcohol, but which, if the extraction were continued long enough, would finally come out and really belong to the alcohol soluble

fraction. The material is again transferred to the cups and extracted for twelve hours with 95 per cent. alcohol. To continue the extractions longer than this is hardly necessary, for, although it is practically impossible to separate completely the constituents of any tissue by extraction with solvents, the following control experiment indicates that the error is not very great.

One hundred grammes of brain material were extracted twice with alcohol and once with ether, as outlined above and described in detail later, and the amounts of sulphur and phosphorus removed estimated. The material was then again ground up and remixed and subjected to another alcohol extraction for twelve hours, and the total sulphur and phosphorus in the extract estimated. The results are as follows:—

4 hours alcohol, 2 hours ether, 12 hours alcohol extractions yielded	...	...	...	...	253.7 mg. BaSO <sub>4</sub>
A further 12 hours alcohol extraction yielded	...	...	...	...	6.5 mg.
					260.2
Percentage extracted in first series	...	...	...	...	97.5

On the same sample phosphorus estimations were made and yielded:—

First fractions	...	...	...	...	...	893.1 mg. Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>
Final fraction	...	...	...	...	...	6.0 " "
						899.1 " "
Percentage extracted in first series	...	...	...	...	...	99.3

Although the total amount of sulphur extracted in the final fraction is only 0.9 mg. as compared with 1.7 mg. phosphorus, it calculates to a little higher in percentage of total on account of the smaller amount of total sulphur extracted with alcohol. The completeness of the extraction is, therefore, fairly satisfactory, especially as the error largely becomes eliminated in a series of comparative experiments such as we are dealing with here.

The residue insoluble in alcohol represents essentially proteins (the amount of glycogen in nerve tissue is too small to be considered) plus a correction for water soluble alcohol insoluble extractives (about 2 per cent. of the total residue). As these extractives, however, are rich in inorganic phosphates and sulphates, it is necessary, after weighing, to extract this residue with hot water. Eight extractions are usually sufficient. In these extractions it is well to exercise great care to avoid the danger of bacterial decomposition, as the results can be completely vitiated by this source of error.

## DETAILS OF METHOD.

One hundred grammes of brain material which have been preserved in 400 c.c. of absolute alcohol and kept with occasional heating to ensure complete coagulation of the proteins for not less than two or more than four months, are transferred to the cups as follows:—The perforations of three cups are covered with filter paper by cutting one round piece to fit the bottom end and one oblong piece to fold against the side. The filter paper is moistened with alcohol and pressed firmly against the sides with a glass rod. Even if all the perforations are covered with filter paper, small particles of brain material will pass through the cracks where the two pieces join, but if the adjustment of the filter paper has been carefully done, these cracks soon fill up and the filtrate is perfectly clear. The cups are then placed in large funnels which drain into beakers placed below and the brain material carefully transferred. It requires three cups to hold 100 grammes of brain material.

At first, as stated above, the filtrate is not clear, but when it begins to come through clear the beakers are changed and the first filtrate refiltered. After all the material has been transferred to the cups in this manner and the last particles washed in with hot alcohol, the cups are allowed to drain and then carefully transferred to the extraction apparatus.

In order to ensure a better distribution of the alcohol, as it drips into the cup from the condenser, a perforated plate is placed on top of the brain material in the cup; the extraction is started with 95 per cent. alcohol at the medium temperature of the electric plate and continued for 3-4 hours. With this temperature the alcohol boils, and about every two seconds one drop falls from the condenser and thus keeps the material thoroughly soaked. After 3-4 hours the apparatus is allowed to cool, clean flasks substituted, and an ether extraction continued for 2-3 hours. The apparatus is again allowed to cool and the material carefully ground up to as fine a powder as possible in an agate mortar. When removing the material from the cups, care must be taken not to disturb the filter paper, as it is to be used again when the material is returned to the cups. The powdering of the material may be a slow and laborious process, but should be carefully and completely done in order to ensure complete extraction later. The powdered material is transferred to a 600 c.c. Jeña flask, moistened with 50 c.c. water and heated on a water bath for one hour. At the end of this time 450 c.c. absolute alcohol are added; the mixture is warmed, well shaken and allowed to stand over-night. The material is again filtered into the cups, as previously described, and extracted for twelve hours with 95 per cent. alcohol. The flasks contain-

ing the alcoholic extracts, the beakers containing the filtrates, and the flasks containing the ether extract are put aside, and their manipulation will be described later under the separation of the lipoids. The residue in the cups is now transferred to a weighed beaker or platinum basin and dried at  $105^{\circ}$ – $110^{\circ}$  C. to constant weight. This weight represents the total protein plus about 2 per cent. of extractives and ash (not removed by the alcohol), and is designated as fraction 3 and 4. It is now necessary to take an aliquot part for the  $S_{3+4}$  estimation. In the dry and very hygroscopic condition of the protein residue this is practically impossible; therefore, after weighing, it is allowed to stand over-night in order to come back into equilibrium with the moisture of the air, it is then again weighed and an aliquot part of one gramme taken for the  $S_{3+4}$  estimation (p. 23). The remainder is transferred to a 300 c.c. Jena flask and moistened with 100 c.c. water; the flask is plugged with a loose cotton wool stopper and heated on a water bath for one hour. This eliminates all risk of bacterial decomposition until next day, when the fluid is filtered into a 600 c.c. Jena beaker. The residue is washed back into the flask and 100 c.c. water again added and the process repeated on each of eight successive days. It is imperative that after the cotton wool stopper has been removed, the material is not kept too long before it is again sterilised. The precautions are merely those which will occur to anyone familiar with bacteriological technique. One point, however, may escape notice. In case it is not possible on account of press of work to start the water extractions the next day after the residue has been dried, it is not safe to allow it to stand. The amount of moisture which is absorbed from the air is sufficient to enable bacteriological growth to commence, and we have frequently noticed samples which, although they were dry when transferred to a dry flask, develop a decided odour of putrefaction on standing. It is advisable in such a case to thoroughly soak the residue in 15–20 c.c. chloroform and carefully stopper it before putting it aside.

The filtrates from the hot-water extractions, as they stand for more than a week before the process is ended, are also liable to bacterial decomposition. The addition of 5 c.c. of conc. hydrochloric acid to the first filtrate avoids this difficulty. When the filtrates accumulate to more than 400 c.c., they must be evaporated so as to keep the bulk at about 300 c.c. The precipitate at first formed when the acid is added to the first filtrate is practically redissolved by this process. A slight amount of a brownish precipitate sometimes forms in the solution, and should be filtered out before the  $S_3$  estimation is made, but not before the total  $S_3$  estimation, as it may contain traces of organic sulphur compounds. The united filtrates, which should not exceed 300 c.c. in

bulk, are then used for the  $S_3^I$  or  $S_3$  and  $P_3$  estimations (pp. 23 & 27). The residue insoluble in hot water is burned by Neumann's method with nitric and sulphuric acids, and used for the  $P_4$  determination (p. 27).

#### SEPARATION OF LIPOIDS.

The alcohol soluble portion (fraction 1 and 2 of the 100 grammes of brain matter) is now contained in three flasks from the first alcohol extraction, three flasks from the ether extraction, two flasks from the second alcohol extraction, and five beakers, three from the first filtrate in which the sample was originally preserved, and two from the treatment after grinding the material in agate mortar. In the first set of these flasks a considerable amount has separated on cooling. The supernatant liquid is carefully decanted off into several 250 c.c. Jena glass evaporating dishes, into which all the other extracts are poured as the evaporation proceeds. The evaporation must be carried on at a low temperature, and in no case must the samples be evaporated to dryness. After they have been reduced to small volume, the residual liquids are allowed to cool and the evaporation finished in a vacuum desiccator. All the alcohol must be removed as completely as possible, as it interferes with the later procedure. After about one week the samples are dry and free from alcohol. They are then moistened with water, allowed to stand over night and the next day transferred to a graduated 1,000 c.c. flask. If the material has been well moistened it can easily be removed from the beakers and extraction flasks as a watery emulsion. After all has been transferred to the litre flask it is well shaken and 10 c.c. conc. hydrochloric acid added. After again shaking 15 c.c. chloroform are added and the bulk made up to 1,000 c.c., well shaken and allowed to stand in a cool place for three to seven days, when a perfectly clear filtrate can be obtained. The danger of decomposition of the lipoids from this acid treatment has already been investigated (1) and occasional control experiments made in the course of the work have confirmed these negative results. If the filtrate is not perfectly clear, it can be shaken up again with chloroform and allowed to stand in a cool place; the solution is then again filtered through a fresh filter paper. In order to obtain a complete separation of the lipoids and the water soluble extractives it would be necessary to wash the lipid precipitate with acid water. This, however, has not been found practicable, but instead the filter paper containing the lipid precipitate is allowed to drain thoroughly and the volume of the filtrate recorded. The difference between this volume and the total volume minus the chloroform approximately represents the amount of liquid adhering to the lipid precipitate. In order to make this figure more

accurate it is necessary to start the filtration through a dry filter paper. The following example will illustrate the case (compare also similar case, p. 24):—

The residue from the evaporation of the total alcoholic extracts of 100 gms. of brain matter were emulsified with water and 10 c.c. HCl and 15 c.c.  $\text{CHCl}_3$  added. The whole was made up to 1,000 c.c., well shaken and allowed to stand five days. The filtrate measured 780 c.c., and yielded on evaporation by the method of sulphur estimation (p. 22) 69.7 mg.  $\text{BaSO}_4$ . The lipid precipitate collected from the filter paper by dissolving in hot alcohol yielded 259.0 mg.  $\text{BaSO}_4$ . Considering that if all the filtrate adhering to the lipoids could have been recovered and allowing for the volume of chloroform, the part of the 205 c.c. difference must be due to the bulk of the lipoids, but if we consider that 100 grammes of brain matter contains only 22 grammes of solids, of which 60 per cent., or 13 grammes, is represented by lipoids, the error is not very considerable. Therefore, we have assumed that the 205 c.c. have the same composition as the other 780 c.c., and that, by calculation, they would yield 18.3 mg.  $\text{BaSO}_4$ , which must be subtracted from the lipid sulphur, leaving 240.7 mg.  $\text{BaSO}_4$ , total  $\text{S}_1$ , and added to the extractive sulphur, making 88.0 mg.  $\text{BaSO}_4$ —total  $\text{S}_2$ . The proportional error in this manner of correction is probably much less than would be incurred by attempting to wash the lipoids.

#### ESTIMATION OF SULPHUR.

##### *Outline of method:—*

In searching for a method suitable for the estimation of sulphur in the moist fatty material with which we are dealing, and in which it is sometimes necessary to estimate accurately 5-10 mg. of sulphur in 2-3 grammes of organic material, the following difficulties must be taken into account:—(a) The possibility of loss from too rapid combustion, especially with dry material, and the danger of spattering with material in a semi-liquid state. (b) The risk of incomplete combustion or oxidation, due to caking of the fusion mixture.

In an excellent review of all the methods Folin (29) finally recommends the sodium peroxide method. This, however, did not prove satisfactory with the moist fatty material we have been investigating, and the method used by Schmiedeberg in his laboratory has been adopted. This method gives very good results in cases where large amounts of organic material have to be destroyed, but it requires much time and care. The principle of the method consists in the gradual charring with an alcohol burner of the material in a mixture of seven

parts sodium carbonate and one part potassium nitrate. This proportion of potassium and sodium avoids the source of error, pointed out by Folin, in the precipitation of the barium sulphate. The final burning is made with a Barthel alcohol burner at a temperature just below the fusion point of the mixture. After acidifying with hydrochloric acid a few drops of bromine water are added to remove any nitrous oxide in the solution, and the precipitation is done in the usual way.

*Description of method*—(a) *Method of fusion*.—One gramme of the material is mixed with at least 12–15 grammes of the fusion mixture ( $\text{Na}_2\text{CO}_3$  seven parts,  $\text{KNO}_3$  one part) in a lipped silver crucible of 30 c.c. capacity, and *very gradually* charred over an alcohol spirit lamp. This process should take several hours, and the fumes from the burning should never rise so rapidly as to discolour very much the top layer of the fusion mixture. Should the fumes come off too rapidly, the spirit lamp must be removed for a short time and fresh fusion mixture sprinkled on the top of the mixture. In this manner the fumes arising from the burning are made to pass through a layer of fresh fusion mixture, and any sulphur fumes are kept back. After the material has completely charred and no more fumes come off even on placing the flame in direct contact with the crucible, the mass after cooling is thoroughly powdered in an agate mortar with the addition of about 1–2 grammes of fresh fusion mixture. It is then returned to the crucible, a layer of fusion mixture sprinkled on top, and heated with the Barthel alcohol burner at a temperature sufficiently low to avoid caking of the fusion mixture. The burning must not be hurried. The combustion will be more complete after heating at a moderately low temperature for half an hour, than by trying to force the process, for any caking is apt to include black specks which are difficult to oxidise, and not only may contain unoxidised sulphur, but also actually reduce some of the sulphates already formed, *e.g.*, the Leblanc soda ash process. The fusion mixture, after cooling, is transferred to a 600 c.c. Jena Erlenmeyer flask, and the crucible boiled out twice with hot water to dissolve any silver sulphate which may have formed through contact with the crucible. If the mixture has caked a little this process of boiling out becomes much more difficult, and almost invariably a black stain of silver sulphide will be seen on the crucible. The loss from this source of error may amount to 1 mg. of  $\text{BaSO}_4$ . The solution with washings is carefully acidified with hydrochloric acid and heated on a water bath to drive off the  $\text{CO}_2$  evolved. It is then filtered and to the boiling filtrate 5–10 c.c. of 10 per cent.  $\text{BaCl}_2$  solution are added and the barium sulphate estimated in the usual manner.

It is usually considered advisable in making sulphate estimations to obtain a weighing of 100 mg. or more of barium sulphate. As this would

involve the destruction of very large amounts of organic material it was decided in these analyses to aim at 40–60 mg. of barium sulphate, considering that the error occasioned by a somewhat smaller weighing would be more than compensated for by the more complete extraction of the material and the decrease in the sources of error accompanying the destruction of large amounts of organic material. The 100 grammes sample of moist brain material recommended in the collection of material accomplishes this.

*Estimation of  $S_1$ .*—The lipoids after filtration following the acid chloroform precipitation, represent a sticky mass adhering to the flask and filter paper. The mass on the filter paper can be washed off with hot alcohol. The destruction of all the lipoids from a 100-gramme sample, however, involves an almost hopeless task, and would yield about 250 mg. barium sulphate. In order to keep the estimation within the limits of accuracy, adopted for the other fractions, it becomes necessary, therefore, to take an aliquot part. This can only be done by dissolving the mass in alcohol in the 1,000 c.c. flask in which the precipitation was originally made and making up to the 1,000 c.c. mark with alcohol; 200 c.c. of this solution should then be used. This procedure, however, is complicated by the fact that the lipoids are only soluble in hot alcohol, which makes the taking of an aliquot part a matter of some difficulty. Before finally adopting this method, therefore, it was necessary to try some control experiments. These were done as follows:—

*Control Exp. I.*—A 50-gramme sample of brain was treated in the usual way, and the lipoids, after dissolving in hot alcohol, made up to 500 c.c. While warm 200 c.c. were taken with a warm pipette and the sulphur estimated. The sulphur in the remaining 300 c.c. of the solution was then also estimated. The yields were 24.0 mg. and 36.0 mg.  $BaSO_4$  respectively, which figures are in the proportion of 200:300.

*Control Exp. II.*—From three 100-gramme samples of three brains the lipoids were made up to 1,000 c.c. and 200 c.c. aliquot parts taken. From these same brains 20-gramme samples of material were collected, and the sulphur in the total lipoids estimated without taking an aliquot part. The following table gives the results:—

CASE No.	BARIUM SULPHATE.		S. CALCULATED IN PER CENT. OF DRY MATTER.			
	Aliquot part.	20 grm. sample.	Aliquot part.	20 grm.		
19	51.8 mg.	43.7 mg.	0.154	0.135		
28	33.4 mg.	31.6 mg.	0.102	0.097		
42	43.5 mg.	42.0 mg.	0.125	0.121		

The agreement is better in the last two samples which were estimated at a later time when more experience had been acquired in taking the aliquot part. There is a tendency for the aliquot part to come out

higher; this can no doubt be accounted for by the cooling of the liquid in the pipette, and must be avoided as much as possible.

*Control Exp. III.*—Seventy-seven grammes of blood which should contain no lipoid sulphur were treated in the same way. All the lipoid precipitate was destroyed and gave no weighable quantity of barium sulphate. Water soluble organic and inorganic sulphates do not, therefore, adhere to the lipoid precipitate in sufficient amount to account for the quantities found. This experiment serves as a negative control.

*Details of method.*—After the lipoid precipitate has been allowed to drain and the volume of the filtrate recorded, the funnel is placed in the litre flask originally used and a hole punched in the bottom of the filter paper with a glass rod. By means of a hot alcohol wash bottle all the sticky mass adhering to the filter paper and to the glass rod is completely washed into the flask. It is better to use 95 per cent. alcohol for this. The amount of alcohol in the flask should now be 400–600 c.c.; the bulk is made up to about 900 c.c. with absolute alcohol and the whole warmed on a water bath until complete solution has taken place. Great care must be taken that the sticky mass, which has a tendency to adhere to the bottom of the flask and which may easily be missed as it is rather transparent, has been completely dissolved. The shaking of the flask must also be carefully done, as too violent shaking may cause the liquid to boil over and thus spoil the analysis. When everything appears to have dissolved, enough warm alcohol is added to make the bulk 1,000 c.c. While keeping the flask on the water bath, a 100 c.c. pipette is now introduced, and by carefully drawing up the liquid and allowing it to again flow back into the flask, at the same time turning the flask, a uniform mixture can be obtained. This treatment at the same time warms the pipette. 200 c.c. are removed as carefully and rapidly as possible, evaporated to a semi-pasty condition and then mixed with fusion mixture.

It is perfectly futile to attempt the destruction of this organic matter with less than 40 grammes of fusion mixture distributed between three silver crucibles of the size previously described. The difficulties experienced at this point will soon convince anyone how practically impossible it would be to try to burn all the lipoid precipitate in this manner instead of taking an aliquot part. There seems to be an unnecessary amount of detail in this description, but we feel convinced that anyone attempting to repeat these analyses with any aim at accuracy would wish there had been more, as the factors which from time to time tended to spoil analyses seemed almost infinite.

*Estimation of  $S_2$ .*—The water solution filtered from the lipoid precipitate is evaporated to moist dryness, mixed with 10 grammes fusion

mixture and the sulphur estimated. In adding the fusion mixture care must be taken to prevent excessive spattering due to the liberation of  $\text{CO}_2$  by the acid present. It is also well *not* to heat this estimation to as high a temperature with the Barthel burner as the others, on account of the fact that the larger amount of sodium chloride present is liable to cause it to cake, also the sodium chloride is liable to attack the silver crucibles and bring about the formation of a colloidal form of silver chloride in the final solution, and this spoils the neatness of the barium sulphate precipitation. It might also be advisable to use a platinum crucible for this estimation.

*Estimation of  $\text{S}_2^1$ .*—The filtrate from a second 100-gramme sample is evaporated to about 300 c.c., filtered, if necessary, and the sulphates estimated directly by barium chloride. The weighings of barium sulphate here amount to about 12.15 mg. Too much confidence must therefore not be placed in this result; it is better to regard it in the nature of a correction, by means of which it is possible to estimate the organic part of the  $\text{S}_2$  fraction. Sulphates appear to be eliminated from the tissues as rapidly as they are formed, so that the amount present at any time is never very great and the variations are of comparatively little significance (3.4 per cent. of total S).

*Estimation of  $\text{S}_4$  and  $\text{S}_{3+4}$ .*—The protein residue insoluble in alcohol is dried, weighed, and allowed to come into equilibrium with the moisture of the air as previously described. One gramme is then taken, mixed with 12 grammes fusion mixture and the sulphur estimated. Great care must be taken *not* to hurry the preliminary burning, as sulphur may be lost on account of the dry nature of the material. The result represents  $\text{S}_{3+4}$ . The remainder of the protein residue is then extracted with hot water (p. 17), the extracts evaporated and mixed with 10 grammes fusion mixture. This result represents the  $\text{S}_1$ , and subtracted from the  $\text{S}_{3+4}$  gives the  $\text{S}_4$  or protein fraction.

*Estimation of  $\text{S}_3^1$ .*—From another 100-gramme sample the eight hot-water extracts of the alcohol insoluble residue are evaporated with 5 c.c. conc. HCl to 300 c.c., filtered if necessary, and the sulphates estimated directly with barium chloride. It is a question whether  $\text{S}_{3+4}$  minus  $\text{S}_4$  or  $\text{S}_{3+4}$  minus  $\text{S}_3^1$  more accurately represents the protein sulphur. The portion of  $\text{S}_3$  of organic nature usually amounts to about 5 per cent. of the total sulphur, and is mainly precipitated by phosphotungstic acid. The amounts, however, are so small that their investigation will be a matter of some difficulty; in recording the analyses in this paper, therefore, the latter figure  $\text{S}_{3+4}$  minus  $\text{S}_3^1$  is invariably referred to as the protein or rather protein-like sulphur.

*Method of keeping analytical records and calculation of results.*—The

method of notation here used has already been explained, and it seemed of interest to describe in a little more detail not only the method of calculating the results but also a method of keeping laboratory notes on the card catalogue system, which may be of general value in investigations of this kind. The copy of a card from one of the cases with analytical results and calculation in logarithms follows:—

CASE 70. C. E. S. Age 43. Dept. of Path. Material collected 5.1.08. Univ. of Chicago. Analysis begun 8.1.08.

100 g. 10 HCl, 15 CHCl <sub>3</sub> , 2 days $\frac{830}{1000}$ filt.	S <sub>1</sub>	S <sub>2</sub>	S <sub>3+4</sub>	S <sub>5</sub>	S <sub>2</sub> <sup>1</sup>	S <sub>3</sub> <sup>1</sup>
S <sub>1</sub> gave (44.1 × 5) mg. BaSO <sub>4</sub>	9129	7642	7267	6435	0769	0969
S <sub>2</sub> „ (58.1 × $\frac{985}{710}$ ) mg. BaSO <sub>4</sub>	1903	8513	1377	1377	8808	1377
	1032	9129	9854	9854	1961	2346
Protein residue weighed 9.671 air dry	3177	1377	8498	7666	1377	0425
S <sub>3+4</sub> 1.000 g. air dry gave 53.3 mg. BaSO <sub>4</sub>	1377	9934	0425	9381	9934	1921
S <sub>5</sub> 8.671 g. „ „ „ 44.0 „	4554	0440	8073	8285	3272	
100 g. 10 HCl, 15 CHCl <sub>3</sub> , 3 days $\frac{760}{1000}$ filt.	0425	0425		0425	0425	
	4129	0015		7860	2847	
S <sup>1</sup> gave 12.0 × $\frac{985}{760}$ mg. BaSO <sub>4</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>3+4</sub>	S <sub>5</sub>	S <sub>2</sub> <sup>1</sup>	S <sub>3</sub> <sup>1</sup>
S <sup>1</sup> gave 12.5 mg. BaSO <sub>4</sub>	25.9%	10%	64.2%	6.1%	1.9%	1.5%

○

○

○

*Explanation of card.*—The first 100 gramme sample was extracted and the alcohol residue after emulsifying precipitated with 10 c.c. HCl and 15 c.c. CHCl<sub>3</sub> and made up to 1,000 c.c. After standing for two days, filtered, and the volume of filtrate 830 c.c. recorded, 710 c.c. of this filtrate were evaporated and gave 58.1 mg. BaSO<sub>4</sub>. The aliquot part of the lipoids (200 c.c. of 1,000 c.c.) gave 44.1 mg. BaSO<sub>4</sub>. The calculation is as follows:—

S <sub>2</sub>		S <sub>1</sub>	
log 58.1 ... ..	7642	log for S <sub>2</sub> in 1 c.c. ... ..	9129
log 710 ... ..	8513	log 155 (filtrate clinging to lipoids). 1903	
S <sub>2</sub> in 1 c.c. ... ..	9129	Correction for S <sub>1</sub> ... ..	1032
log BaSO <sub>4</sub> to S ... ..	1377	Antilog for correction ... ..	12.7 mg.
log 985 (total filtrate) ... ..	9934	(44.1 × 5) — 12.7 = ... ..	207.8 mg.
	0440	log 207.8 (actual S after corr.) ... ..	3177
log for total S ... ..	0427	log BaSO <sub>4</sub> to S ... ..	1377
S <sub>2</sub> in % of total S ... ..	0013		4554
Antilog ... .. 10.00%		log for total S .. ..	0427
		S <sub>1</sub> in % of total S .. ..	4127
		Antilog ... ..	25.9 %
S <sub>3+4</sub>		Calculation of total S.	
log 53.3 ... ..	7267	Antilog 0440 ... ..	1107
log BaSO <sub>4</sub> to S .. ..	1377	„ 4554 ... ..	2852
log 9.671 ... ..	9854	„ 8498 ... ..	7076
	8498		
log for total S ... ..	0427		11035
log S <sub>3+4</sub> ... ..	8071		
Antilog ... .. 64.2%		log 11035 (total S) is 0427.	

The other calculations follow on the principles here outlined, and should be easily understood.  $S_2^I$  and  $S_3^I$  were done on second 100-gramme sample. It will be evident that by thus expressing the results in per cent. of total sulphur the water estimation does not need to be taken into account. In several cases the total S in per cent. of dry tissue was estimated directly on a sample of brain matter and found to check that obtained by calculation from the different fractions. The phosphorus estimations were recorded in similar ways on cards of a different colour, and were calculated in practically the same way as here described.

### ESTIMATION OF PHOSPHORUS.

*Outline of method.*—In order not to multiply needlessly the work, a method of estimating phosphorus was adopted which could be applied to the same material used for the sulphur estimations. For this purpose the filtrate from the  $BaSO_4$  precipitate, which should contain all the phosphorus as phosphoric acid, was treated with ferric chloride and ammonia. In order to test if this method removed all the phosphorus as iron phosphate, controls were made with the filtrates, and these indicated that the precipitation has to be repeated in order to ensure complete removal. The following results will illustrate this:—

Case.		First Fe ppt. contained		Second Fe ppt. contained
42	$P_1$	..	148.8 mg. $Mg_2P_2O_7$ ,	8.1 mg. $Mg_2P_2O_7$ ,
15	$P_2$	..	65.4 " "	5.6 " "
15	$P_1$	...	143.4 " "	12.9 " "
42	$P_2$	..	94.8 " "	13.8 " "

The use of a greater amount of iron in the first place does not obviate this difficulty. Another source of error in this method lies in the fact that on account of the large amount of iron used, the magnesium pyrophosphate precipitates are very apt to show a slight iron stain. No amount of washing or control of the amount of iron can obviate this, as the iron seems to enter into the complex molecule of the ammonium phosphomolybdate as a component part. However, the following results indicate that the error is not great and is well within the variations of the material:—

Case.		$Mg_2P_2O_7$ mg.		Fe		Percentage error.
19	$P_1$	....	92.4	1.4	....	1.4
30	$P_2$	.....	144.2	1.3	.. ...	1.0
70	$P_2$	.....	169.8	2.5	....	1.5
29	$P_2$	.....	207.7	1.0	.....	0.5
70	$P_4$	.. ...	57.4	0.5	..	1.0

The iron was estimated colorimetrically with KCNS by comparison with an iron solution of known strength. It is not necessary to enter into the

details of this method here as the usual errors would not very seriously affect the above observations.

*Details of method.*—To filtrate from  $\text{BaSO}_4$  precipitation add from 1–3 c.c. of a 20 per cent. ferric chloride solution. Add 5–10 c.c. ammonia, sufficient to make an excess, boil until the fumes of ammonia are no longer given off and then filter. To the filtrate add again 1 c.c. ferric chloride solution and repeat precipitation. Place the funnel containing the two precipitates in the flask in which the precipitation has been made, punch a hole in the bottom of the filter paper with a glass rod, and wash the precipitate into the flask with hot water. The filter paper is then moistened with 1 c.c. nitric acid and washed until all traces of iron are removed. Another cubic centimetre of nitric acid is usually sufficient to dissolve the whole precipitate. It is necessary to effect this transference while the precipitate is quite moist, as it becomes hard and insoluble in nitric acid on standing. In the  $P_1$  and  $P_2$  estimations it is desirable at this point to filter the solution and to take an aliquot part (e.g., 150 of 250 c.c.), for the amount of phosphorus in these fractions is comparatively large, and the precipitates obtained are too bulky to manipulate neatly; also good re-agents may be wasted unnecessarily.

In this solution the phosphorus is estimated in the usual way (30) with nitro-molybdate solution in the presence of an excess of ammonium nitrate. All estimations recorded in this communication were finally weighed as  $\text{Mg}_2\text{P}_2\text{O}_7$ .

*Estimation of  $P_1$ .*—This fraction is estimated in the filtrate from the  $S_1$  precipitation, and a correction applied for  $P_2$  in the same manner as in the  $S_1$  calculation. The method devised by Köch and Woods (30) for the separation of lecithin and kephalin has been found to vary so much with the conditions of the experiment that the results seem hardly reliable.

*Estimation of  $P_2$ .*—This estimation is made on the filtrate of the  $S_2$  barium sulphate precipitation. With regard to the estimation of  $P_2^1$  the results have been uncertain. Grindley (31) has attempted the separate estimation of inorganic and organic combined phosphates, but it does not appear to us at all certain that his method may not break up organic radicles. One experiment was made to estimate the phosphates directly in this fraction with magnesia mixture. The results are as follows:—

	$P_2$ BY FUSION IN PER CENT. OF TOTAL P.	$P_2^1$ DIRECT BY MAGNESIA MIXTURE.
Case 26	11.4	5.2

There does appear to be present a considerable amount of organically combined phosphates which are not broken up by this method.

*Estimation of  $P_3$ .*—This fraction seems to consist entirely of  $P_3^i$  inorganic phosphates, unless we are here again dealing with the breaking up of organic radicles:—

	PHOSPHORUS BY FUSION.	P. BY DIRECT PITT WITH NITRO-MOLYBDATE.
Case 26	9.8	9.9
Case 70	10.6	10.5

In recording the results  $P_2$  and  $P_3$  are added together under the term extractive phosphorus.

*Estimation of  $P_4$ .*—The residue after extraction with hot water is burned by Neumann's method and the phosphorus estimated in the usual way. The very great variations observed in the results obtained by this method, still further emphasise the difficulties of estimating milligrammes of phosphorus in grammes of organic material.

#### ESTIMATION OF WATER.

The amount of blood in the tissues at death and the accidental and almost uncontrollable variations in the amount of drying during the collection of the material are apt to introduce differences in the water content of various brains, and for these reasons not much importance has been attached to the comparatively slight variations which occur with some normal and pathological brains. It is, however, necessary to estimate the water content in an investigation of this kind in order to determine the various constituents in percentage of the total solids.

It has been already demonstrated (1) that it is difficult to dry to constant weight material of colloidal nature, and that the best method consists in drying in vacuo below the coagulation point of the colloid. This method has been adopted. Weighed quantities of minced brain matter have been spread out on one of two tared (paired) watch glasses fitted with a clip, allowed to remain in a vacuum desiccator for some time and then dried to constant weight in a vacuum oven at  $40^{\circ}$ – $42^{\circ}$  C. When constant weight is attained, it is found that raising the temperature to  $100^{\circ}$  C. does not materially affect the result.

Benedikt (32) has since devised a method which in principle resembles our method, but offers no special advantages.

The amount of material taken should be as near as possible 2 grammes; a larger quantity than this makes the drying a very long and tedious operation, while smaller amounts on account of their smallness may introduce error. A number of moisture determinations made in duplicate are given below, and the results not only show a fair check of accuracy of the method, but also indicate that the mincing process gives a uniform mixture of the grey and white matter, as the samples

were taken in each instance from quite distinct portions of the minced material:—

NO. OF CASE.		28.	40.	41.	29.	42.
Per cent. of moisture	{ (a)	78.61	77.41	78.38	77.52	76.68
	{ (b)	78.74	77.50	78.55	77.49	76.83

#### ESTIMATION OF GROUPS OF CONSTITUENTS.

*Proteins, extractives and ash.*—The method is essentially that outlined before (1). The proteins are calculated by subtracting the weight of the residue from the hot-water extractions from the original dry weight of the alcohol insoluble residue. The extractives are made up from the above water soluble residue and the residue from the filtrate derived from the lipid precipitation corrected for total volume. The ash is the residue obtained on incineration of the extractives at dull red heat. Not many observations are recorded in these pages, as they did not appear to show any great variations.

*Cerebrins.*—This estimation is accomplished by determining gravimetrically with Fehling's solution the amount of galactose split off with dilute HCl.

When the aliquot part of the lipid precipitate of 100 grammes brain matter is being taken for the  $S_1$  estimation another 100 c.c. (*i.e.*, one-tenth aliquot part) is taken for the cerebrin estimation. This is placed in a *litre flask* and the alcohol and chloroform *completely evaporated*. Even then, it is well to add a little distilled water and to boil for some time, for the presence of alcohol and chloroform is liable to give rise to other reducing compounds, *e.g.*, phosgene, and thus vitiate the result. A large flask is necessary, for the frothing is rather considerable, and unless this precaution is heeded will cause much annoyance. The bulk of the solution is made up to 100 c.c. and 3 c.c. HCl added. The flask is then fitted with a reflux condenser, placed on a sand bath and gently heated for at least 24 hours, not necessarily consecutive. Towards the end of the hydrolysis the solution may appear milky; it is advisable then to add a few more drops of conc. HCl, when this will disappear. The solution with washings is now transferred to a 250 c.c. graduated flask; sodium sulphate, solid or in concentrated solution, is added until all precipitation is complete and the supernatant fluid is clear and bright, then the bulk is made up to 250 c.c., the flask well shaken, and allowed to stand for a while. The solution is then filtered and an aliquot part 100 150 c.c. taken; to this, after carefully neutralising, is added 100 c.c. freshly-made Fehling's solution, and the whole stirred and placed on a water bath for four hours. At the end of that time the precipitate is collected on a Gooch asbestos filter, washed with hot distilled water, ignited and

weighed as  $\text{CuO}$ , or reduced further and weighed as metallic copper. From this weighing the equivalent amount of galactose is ascertained, and an approximation of the amount of cerebrin obtained by multiplication with 100/21.8, Thierfelder's factor for the sugar equivalent of cerebrin. The Fehling's solution must always be in considerable excess, and during the precipitation the solution should not be allowed to evaporate to any great extent.

Sugar determinations have been made on the filtrates obtained from the lipoid precipitation. In each instance, after boiling off the chloroform present, no reduction occurred.

*Cholesterin.*—The method of Ritter (33) was again used in the few cholesterin determinations made in this work.

### III. ANALYTICAL RESULTS.

The above methods permit the comparison of normal and pathological material from three points of view:—

1. *Condition of nutrition of tissue.*—This considers the variations in the three principal food constituents; proteins, fats (lipoids) and carbohydrates (cerebrin).

2. *Changes in oxidations.*—It will be very difficult to draw any conclusions with regard to the intensity of oxidations from any variations in the sulphates. It appears that the sulphates, like carbon dioxide and urea, are very rapidly eliminated from the tissues and soon appear in the urine. As a result they are only found in the tissues in amounts too small to permit any conclusions based on variations (15 mg.  $\text{SO}_4$  in 100 grammes brain material). To attempt to study the oxidations by the difference in composition of the blood going to and coming from the brain, such as Hill (34) has attempted with carbon dioxide, would be quite out of the question with material which cannot be obtained until after death. It remains, therefore, to compare variations in the intermediary oxidation compound which appears to have a greater affinity for some constituents of the protoplasm, and is hence not eliminated so rapidly, *i.e.*, the taurin-like compound described by one of us (13), here referred to as neutral sulphur.

3. *Destructive changes accompanied by reparative growth.*—These changes it is proposed to study by the variations in the phosphorus. An increase in extractive phosphorus would thus indicate a period of increased growth, together with a greater supply of material from which to build up the more complex phosphoric acid derivatives. This increase in extractive phosphates may be due to increased food supply, or during destructive changes to increased breaking down of complex derivatives.

It is necessary to bear in mind, in looking over the results of these methods, that they only express a relative change. Thus if we find that the total phosphorus of a brain of a general paralytic expressed in percentage of dry matter is the same as that of the normal, it is not correct to conclude that such a brain has not lost phosphorus as the result of the destructive changes which we know have taken place. It merely means that the relative proportion has not changed. In expressing the results for the various groups in the sulphur and phosphorus derivatives, it seemed better to give them in percentage of total phosphorus and sulphur. This eliminates a number of errors and permits of a very good comparison of the different samples with one another, besides enabling the comparison of these results with analyses made by other investigators. A great many interesting investigations of pathological material are vitiated by lack of attention to this point. The system of notation used in the analytical work and there explained is here translated into terms which are more familiar:—

*Protein sulphur* represents  $S_{1+2}$  minus  $S_3^1$ .

*Lipoid sulphur* represents  $S_1$ .

*Neutral sulphur* represents  $S_2$  minus  $S_3^1$ .

*Inorganic sulphur* represents  $S_2^1$  plus  $S_3^1$ .

An explanation may not be out of place for the term neutral sulphur. It represents a combination of sulphur which does not split off sulphuric acid on prolonged boiling with hydrochloric acid, neither does it form lead sulphide on boiling with alkali and lead acetate. The term neutral is used in contradistinction to sulphates or sulphuric acid, and represents merely a makeshift until the chemical nature of this compound or group of compounds can be more definitely established.

Phosphorus group:—

*Protein phosphorus* represents  $P_4$ .

*Lipoid phosphorus* represents  $P_1$ .

*Extractive phosphorus* represents  $P_2$  plus  $P_3$ .

It seems much better to express the results in this manner than to attempt to calculate protein phosphorus into nucleo-proteins or lipoid phosphorus into lecithin, until we know a great deal more of the chemical nature of these compounds, which at present can only be designated as rather indefinite groups. In a few tables for better comparison the lecithin and kephalin were occasionally calculated from the lipoid phosphorus by multiplication with the factor 25.77.

The word *extractive* in these investigations is used on the old basis originally intended by Liebig when he used the term. It has become the custom of some investigators to refer to lipoid phosphorus as extractive,

because it is extracted with alcohol. This is incorrect, and only brings confusion to the term originally used.

*Changes with age.*—We will first consider the variations in the relative composition of the nervous system at different ages. A preliminary report of this subject we gave to the Physiological Society (25):—

## COMPARISON OF BRAINS AT DIFFERENT AGES.

TABLE I.

	CASE 13	CASE 14.		CASE 15.	
	Age, 6 weeks ♀ Weight, 640 grammes.	Age, 2 years ♀ Weight, 1100 grammes		Age, 19 years ♂ Weight, 1670 grammes.	
	Whole brain.	Cortex.	Corpus. callosum.	Cortex.	Corpus. callosum.
Proteins ...	46.6	48.4	31.9	47.1	27.1
Extractives ..	12.0	10.0	5.9	9.5	3.9
Ash ..	8.3	5.8	3.2	5.9	2.4
Lecithins and kephalins	24.2	24.7	26.3	23.7	31.0
Cerebrins	6.9	8.6	17.2	8.8	18.3
Lipoid S. as SO <sub>4</sub>	0.1	0.1	0.5	0.1	0.5
Cholesterin (by diff.)	1.9	2.4	15.0	4.9	16.8
Total S.	0.52	0.53	0.63	0.46	0.50
Total P. ..	1.72	1.50	1.46	1.45	1.45
Moisture .. ..	88.78	84.49	76.45	83.17	69.67

*Distribution of Sulphur in per cent. of total S.*

Protein S. ...	62	63	55	76.5	56
Lipoid S. ...	6	6	27	7	36
Neutral S. ...	26	22	13	10	5
Inorganic S.	6	9	5	6	3.5

*Distribution of Phosphorus in per cent. of total P.*

Protein P. ...	5	6	6	5	5
Lipoid P. ...	54	62	72	63	81
Extractive P. ...	41	32	22	32	15

Two additional cases were studied, and are given in the following table compared with Case 13:—

TABLE II.

	CASE 13.	CASE 19.	CASE 70.
	Age, 6 weeks ? Weight, 640 grammes.	Age 24 years ♂ Weight, 1230 grammes	Age 43 years ♂ Weight, 1400 grammes.
	Whole brain.	Whole brain.	Whole brain.
Protein S. . . . .	62	60	62·5
Lipoid S. ... ..	6	27	26
Neutral S. ... ..	26	9·5	8
Inorganic S. ... ..	6	3	3·5
Protein P. ... ..	5	4	6
Lipoid P. ... ..	54	73	75
Extractive P. ... ..	41	23	19

In the above tables is to be observed with the growth of the brain:—

1. A decrease in moisture, proteins, extractives, and ash, a change usually found in growing tissues.

2. An increase in cerebrin, lipoid-sulphur and cholesterin; in other words, the substances which predominate in the white matter. As far as they go, our results on the younger brains are in harmony with the work of Kaes (35) on the influence of age on the myelination of fibres, but the number of cases, especially senile cases, is not sufficient to permit of any close correlation with his observations.

In connection with the changes due to age, it seems of interest to compare a brain of lower anatomical development. A comparison of the brain of the dog and human follows:—

TABLE III.

(COMPARISON OF BRAIN OF HUMAN AND DOG.

	DOG (1). Weight... 75 grammes. Whole brain.	HUMAN (Case 19). 1,230 grammes. Whole brain.
Protein S. ... ..	73	60
Lipoid S. ... ..	18	27
Neutral S. ... ..	6	9·5
Inorganic S. ... ..	3	3
Protein P. ... ..	4·5	4
Lipoid P. ... ..	70	73
Extractive P. ... ..	25·5	23
MOISTURE ... ..	78·1	77·9
TOTAL P. ... ..	1·50	1·50
TOTAL S. ... ..	0·45	0·50

The agreement in the phosphorus is quite close. The variation in the lipid and neutral sulphur will be discussed at a later date when an investigation of a series of brains from different animals which is to be undertaken by one of us, has been completed.

*Changes involved by reason of the nature of the cause of death:—*

The variations that may be introduced by *post-mortem* change have already been discussed (p. 12). As a good many of the mental cases here studied died of tuberculosis, it appeared of interest to compare three normal brains, one of which was from a case of tuberculosis, the others from cases which had died of other causes.

TABLE IV.—COMPARISON OF BRAINS OF CASES DYING FROM DIFFERENT CAUSES.

Cause of Death...	CASE 15.		CASE 19.			CASE 70.
	<i>Slow Haemorrhage</i>		<i>Tuberculosis.</i>			<i>Diffuse suppurative meningitis.</i>
	Cortex.	Corpus callosum.	Cortex.	Corpus callosum.	Whole brain	Whole brain.
Protein S. . . . .	76.5	56	75.0	54	60	62.5
Lipoid S. . . . .	7.0	36	7.0	36	27	26
Neutral S. . . . .	10.0	5	12.0	6.5	9.5	8
Inorganic S. . . . .	6	3.5	5.5	3	3	3.5

In spite of the great difference in the cause of death (a chronic, an acute infection, and a simple loss of blood) the results are fairly uniform, and are within experimental error. They also indicate that variations in the amount of blood in the brain at death do not introduce an appreciable error.

#### CHANGES OBSERVED IN MENTAL CASES.

(a) The cases chosen were those in which a clinical diagnosis of dementia præcox was given. The results are as follows:—

TABLE V.—VARIATIONS IN THE PROXIMATE CONSTITUENTS.

	Cortex		Corpus callosum.	
	CASE 15 Normal	CASE 17. Dementia Præcox.	CASE 15. Normal.	CASE 17. Dementia Præcox.
Protein ... ..	47.1	49.4	27.1	27.8
Extractives ... ..	9.5	7.7	3.9	3.0
Ash ... ..	5.9	5.5	2.4	2.7
Lecithin and Kephâlin ..	23.7	23.0	31.0	29.7
Cerebrins ... ..	8.8	9.3	18.3	20.2

The only change of any magnitude involves a decrease of the extractives to which attention has already been drawn by one of us (36) in a previous paper.

TABLE VI.

COMPARISON OF CORTEX AND CORPUS CALLOSUM OF TWO NORMAL BRAINS AND TWO BRAINS OF DEMENTIA PRÆCOX.

	NORMAL			DEMENTIA PRÆCOX.			AVERAGES		PERCENTAGE VARIATION FROM NORMAL.
	Case 15.	Case 19.	Percentage variation.	Case 17.	Case 30.	Percentage variation.	Normal.	Dementia Præcox.	
<i>Cortex.</i>									
Protein S. ...	76.5	75.2	1.6 per cent.	81.1	77.3	4.2 per cent.	75.8	79.2	+ 4.5
Lipoid S.	7.0	7.1	0.0	8.7	9.2	5.5	7.1	8.9	+ 25.7
Neutral S. ...	10.0	12.2	20.0	5.4	8.7	47.0	11.1	7.0	- 37.3
Inorganic S.	6.4	5.4	16.6	4.7	4.7	0.0	5.9	4.7	- 20.0
<i>Corpus Callosum.</i>									
Protein S. ...	55.8	54.1	3.1 per cent.	58.0	55.6	4.2 per cent.	54.9	56.8	+ 3.5
Lipoid S. ...	36.1	35.9	0.0	35.6	37.4	5.0	36.0	36.5	+ 1.4
Neutral S. ...	4.6	6.5	34.5	2.7	3.2	16.6	5.5	3.0	- 45.4
Inorganic S.	3.5	3.3	6.0	3.8	3.8	0.0	3.4	3.8	+ 11.7

TABLE VII.  
COMPARISON OF THE CHEMICAL COMPOSITION OF THE WHOLE BRAIN FROM TWO NORMAL CASES AND FOUR CASES OF DEMENTIA PRÆCOX.

	NORMAL.			DEMENTIA PRÆCOX.					AVERAGES.		Percentage variation from normal.
	Case 19 (Case 20)	Percentage variation.	Case 28	Case 29	Case 41.	Case 42.	Maximum percentage variation.	Normal.	Dementia Præcox.		
Protein S . . .	60.2	55.8	7.6 per cent.	64.7	59.7	65.0	66.7	10.9 per cent.	58.0	64.0	+ 10.3
Lipoid S . . .	27.1	32.1	16.6 "	24.6	28.8	23.0	24.8	23.2 "	29.6	25.3	— 14.0
Neutral S . . .	9.5	8.8	7.7 "	5.0	5.6	6.1	3.3	56.0 "	9.2	5.0	— 46.7
Inorganic S . . .	3.3	3.3	0.0 "	5.6	5.9	5.9	5.2	12.5 "	3.3	5.6	+ 40.0
Total S in per cent. of dry matter . . .	...	...	.	...	...	.	...	...	0.48	0.48	0.0
Protein P . . .	3.7	4.7	25.0 "	3.8	5.1	3.6	4.7	35.0 "	4.2	4.3	0.0
Lipoid P . . .	73.1	70.3	3.9 "	73.7	73.0	73.6	70.8	4.0 "	71.7	72.8	+ 1.5
Extractive P . . .	23.2	25.1	8.0 "	22.6	21.9	22.9	24.4	10.9 "	24.1	22.9	— 5.2
Total P in per cent. of dry matter . . .	...	...	.	...	...	.	...	...	1.50	1.42	— 5.0

The largest percentage variation is found in the neutral sulphur. It varies in the cortex of the two cases of dementia præcox among themselves, but in each instance it is lower than in the normal cortex. The variations in the inorganic sulphates are of little value on account of the small amounts, the estimations amounting in reality to a correction only. The proportionate amounts of neutral sulphur are not much greater, but in order to confirm the results analyses were made on 100-gramme samples of a minced hemisphere.

The variation in the total sulphur and phosphorus are within the limits of error. The largest variation again is the decrease of neutral sulphur, and the next largest the increase of sulphates. The increase in sulphates may not necessarily be of significance, as the actual quantities were very small and consequently liable to analytical error; this, however, was not the case in the neutral sulphur estimations. Although the variations in the neutral sulphur of the pathological cases among themselves are considerable, they are all in the same direction, and consequently of a consistent nature.

The absence of variations in the phosphorus fractions is striking.

(b) *Dementia paralytica*.—This form of insanity was selected to serve as a control of the analytical technique of the adolescent cases:—

TABLE VIII.  
VARIATIONS IN THE PROXIMATE CONSTITUENTS.

	CORTEX		CORPUS CALLOSUM.	
	Case 15 Normal.	Case 16 G. P.	Case 15 Normal.	Case 16 G. P.
Proteins . . . . .	47.1	50.7	27.1	30.0
Extractives . . . . .	9.5	7.6	3.9	3.7
Ash . . . . .	5.9	6.0	2.4	3.2
Lecithins and kephalins . . . . .	23.7	23.9	31.0	25.0
Cerebrins . . . . .	8.8	9.4	18.3	18.2

There is an increase in proteins probably correlated to the growth of glia tissue, a decrease in extractives in the cortex and a very marked decrease in the lecithin and kephalin of the corpus callosum. The latter decrease is not so well demonstrated in the following cases, in which uniform samples of a whole hemisphere were analysed. However, there is a tendency for the lipid phosphorus to be decreased.

# CHEMICAL EXAMINATION OF BRAIN

TABLE IX.

COMPARISON OF THE CHEMICAL COMPOSITION OF THE BRAINS OF TWO NORMAL CASES AND FOUR CASES OF GENERAL PARALYSIS.

	NORMAL			GENERAL PARALYSIS.					AVERAGES		Percentage variation from normal.
	Case 18.	Case 70	Percentage variation.	Case 22	Case 23.	Case 24	Case 40	Maximum percentage variation	Normal.	General paralysis.	
Protein S	60.9	62.6	2.7 per cent.	56.7	57.1	62.0	61.4	9.0 per cent.	61.7	59.3	— 3.9
Lipoid S	28.6	25.9	10.0	30.2	30.8	25.1	28.4	21.0	27.2	28.6	+ 5.2
Neutral S	7.2	8.1	11.5	9.3	8.3	9.1	6.4	35.0	7.7	8.3	+ 8.0
Inorganic S	3.3	3.4	3.0	—	3.7	—	—	—	3.4	3.7	+ 9.0
TOTAL S in per cent. of total solids	...	...	...	...	...	...	...	...	0.50	0.48	— 4.0
Protein P	3.9	5.7	38.0 per cent.	5.9	4.8	4.6	4.6	26.0 per cent.	4.8	5.0	+ 4.0
Lipoid P	71.8	75.0	4.4	69.3	70.6	68.2	69.9	3.4	73.4	69.5	— 5.3
Extractive P	24.2	19.4	21.6	24.7	24.7	26.9	25.6	8.5	21.8	25.5	+ 17.0
TOTAL P in per cent. of total solids	...	...	...	...	...	...	...	...	1.45	1.46	0.0

The variations from the normal here are remarkably slight. It is especially interesting to note that, with one exception, the neutral sulphur is not decreased. This distinguishes these cases from those of dementia precox, and also serves as a control of the analytical technique. Although the lipoid phosphorus has a decided tendency to be decreased with a corresponding increase of the extractive phosphorus, the variation is not so striking as might be expected. The increase in nuclein phosphorus observed by one of us (W. K.) and Goodsen (37) is apparently not present in all cases.

(c) *Other cases of mental disorder.*—As a further test of the methods two mental cases with the clinical diagnosis of melancholia were taken for analysis. The results are given in the following table:—

TABLE X.

COMPARISON OF THE BRAINS OF TWO NORMAL CASES AND TWO CASES OF MELANCHOLIA.

	NORMAL.		MELANCHOLIA.		AVERAGES		PERCENTAGE VARIATION.
	Case 18.	Case 70.	Case 25.	Case 26.	Normal.	Pathological.	
Protein S. . .	60.9	62.6	57.4	56.9	61.7	57.2	- 7.0
Lipoid S. . . .	28.6	25.9	29.7	32.4	27.2	31.0	+13.9
Neutral S. . .	7.2	8.1	9.3	7.0	7.7	8.1	+ 5.2
Inorganic S.	3.3	3.4	—	—	3.4	—	—
Protein P. . .	3.9	5.7	4.2	4.1	4.8	4.2	- 15.0
Lipoid P. . . .	71.8	75.0	69.2	74.7	73.4	72.0	- 2.0
Extractive P. .	24.2	19.4	26.6	21.2	21.8	23.9	+10.0

The variations are comparatively slight, and permit of no conclusions that would not be vitiated by differences due to the material or the analytical technique. The sulphates were not estimated in the two pathological cases, and the neutral and protein sulphur correction was applied according to the results of other cases.

#### EXPERIMENTAL CHANGES (with Dr. F. H. Pike).

As the results in dementia præcox might be interpreted as representing a reduced oxidation, some experiments were undertaken with Dr. F. H. Pike, of the University of Chicago, to study the effect of cutting off the four arteries (two carotids and two vertebrals) from the brain of a dog. As Mott and Hill have shown (38), if the animal recovers at all from the operation the recovery is complete. Such proved to be the case in the cases of which the analyses are given in the following table:—

TABLE XI.

	Dog (2)		Dog (3)	
	Weight of brain 46.08 grammes.		51.38 grammes.	
	3 days after operation.		2 months after operation.	
Protein S. . . .	...	73.9	.....	73.9
Lipoid S. . . .	...	16.1	...	16.0
Neutral S. . . .	...	7.1	.....	6.9
Inorganic S. . .	...	2.9	.....	3.2

The first case serves as a control, for it is hardly likely that an organ which has such a special metabolism as the brain would change in a few

days. The results show no variation at all, and give a very good idea of the accuracy to be expected from the methods outlined in this paper.

#### IV. DESCRIPTION OF CASES.\*

*Case 13.*—S. H., Evelina Hospital, London, 7/11/06. Female; age six weeks. Autopsy 24 hours after death.

*Weight of brain.*—640 grammes. White matter had not differentiated sufficiently to be capable of microscopic separation. Whole right hemisphere, after removing basal ganglia, used for chemical analyses. On account of the premature birth this brain is very much under developed for its age.

*Cause of death.*—Prematurely born; died from inanition.

*Case 14.*—E. McC., Evelina Hospital, London, 8/12/06. Female, age 1 year 11 months. Autopsy ten hours after death.

*Weight of brain.*—1,100 grammes.

*Cause of death.*—Exhaustion following (two days after) operation for umbilical hernia. No evidence of peritonitis.

*Case 15.*—R. A. G., Dept. of Path. Univ. of Chicago. 199 M. March 7th, 1907. Male, age 19 years. Autopsy seven hours after death.

*Weight of brain.*—1,670 grammes. No wasting; no excess of fluid. Very anæmic. The Betz cells are perfectly normal. Many of the cells of the other cortical layers are somewhat swollen (cell body and nucleus), and show slight chromatolysis. The change is similar to the one observed experimentally with lack of oxygen. In no case, however, is there any gross change. The cells of the prefrontal area appear to be normal.

*Cause of death.*—Hæmorrhage from right internal carotid artery, following erosion by peritonsillar abscess. Death occurred as a result of continuous bleeding two days after onset.

*Mental state.*—Normal and of good order of intellect

*Previous occupation.*—Student in high school.

*Case 16.*—C. T., Claybury Asylum. 46.M.06/2. Male, age 46 years. Autopsy 24 hours after death, body kept in cold chamber.

*Weight of brain.*—1,230 grammes. Much wasting, large excess of fluid. In the prefrontal region great destruction of nerve cells. Vessels greatly thickened. Marked neuroglia proliferation and all characteristic changes of general paralysis.

*Cause of death.*—Acute pulmonary tuberculosis.

\* Numbers in continuation of cases previously published.

*Diagnosis of mental state.*—General paralysis of the insane, with progressively slow dementia.

*Previous occupation.*—Labourer.

*Length of time in Asylum.*—Seven months.

*Case 17.*—H. F. R., Claybury Asylum. 36.M.06/2. Male, age 20 years. Autopsy nine hours after death.

*Weight of brain.*—1,645 grammes. Excess of cerebrospinal fluid (S=18 parts per million; P=160 parts). Some wasting. The nerve cells (except the Betz cells) throughout the cortical layers in ascending frontal and parietal region, except for a little swelling, are normal. Most of the Betz cells in the ascending frontal are somewhat swollen and show definite chromatolysis, most often perinuclear. Some of the Betz cells are quite normal. The neuroglia cells are swollen and show some evidence of recent proliferation.

*Cause of death.*—Pulmonary tuberculosis.

*Diagnosis of mental state.*—Dementia Præcox.

*Previous occupation.*—French polisher.

*Length of time in Asylum.*—2½ years.

*Case 18.*—A R., Charing Cross Hospital, London. M./07. Male, age 49 years. Autopsy 17 hours after death.

*Weight of brain.*—1,270 grammes. No wasting. Nerve cells normal. Some proliferation of the blood vessel walls.

*Cause of death.*—Pulmonary tuberculosis.

*Diagnosis of mental state.*—Normal.

*Previous occupation.*—Commission agent.

*Case 19.*—W. D., Brompton Hospital, London. M./07. Q.p.31. Male, age 24 years. Autopsy 19 hours after death.

*Weight of brain.*—1,230 grammes. Convolutional pattern good. No wasting. Most of the nerve cells are normal, although a considerable number show chromatolysis, without much destructive change. Some Betz cells show chromatolysis which is generally perinuclear, and resembles that observed in dementia præcox. There is some slight vascular and neuroglia proliferation.

*Cause of death.*—Pulmonary tuberculosis. Duration of disease 18 months, in hospital three months.

*Diagnosis of mental state.*—Normal.

*Previous occupation.*—Printer.

*Case 20.*—A. C. C., St. Thomas's Hospital, London. M./07. Male, age 16 years. Autopsy 30 hours after death, body kept in cold chamber.

*Weight of brain.*—1,440 grammes. No wasting. The nerve cells in

pyramidal layer are very irregularly arranged, a fair number show various stages of chromatolysis, but most are of normal appearance. There is also some evidence of destructive change in the cells of the pyramidal layer. The Betz cells show less change than those of the previous case. The vessel walls are thickened, and there is a moderate amount of vascular and neuroglia proliferation (more than in Case 19). The pia is swollen and thickened and the vessels of the pia and cortex generally are congested. On account of the histological appearance the case might be mistaken for early G. P. (Dr. Helen Stewart).

*Cause of death.*—Tuberculosis of lungs and intestines. Two months in hospital. History of tuberculosis in family.

*Diagnosis of mental state.*—Normal. Physiognomy of a low type.

*Previous occupation.*—Hall boy.

*Case 22.*—E. J. B., Claybury Asylum. 123.M.07. Male, age 37 years. Autopsy four hours after death.

*Weight of brain.*—1,190 grammes. Microscopic examination revealed characteristic changes of general paralysis.

*Cause of death.*—Exhaustion of G. P. I. Congestion and œdema of lungs.

*Diagnosis of mental state.*—General paralysis of the insane.

*Previous occupation.*—Plumber.

*Length of time in Asylum.*—Fifteen months.

*Case 23.*—M. A. P., Claybury Asylum. 5.F.0712. Female, age 40 years. Autopsy 17 hours after death.

*Weight of brain.*—1,055 grammes. Some general wasting. Microscopic examination revealed characteristic changes of G. P. I.

*Cause of death.*—Acute pulmonary tuberculosis.

*Diagnosis of mental state.*—General paralysis of the insane.

*Previous occupation.*—Fur hand.

*Length of time in Asylum.*—Two years nine months.

*Case 24.*—C. B., Claybury Asylum. 6.F.07/2. Female, age 44 years. Autopsy one hour after death.

*Weight of brain.*—1,055 grammes. Considerable wasting. Microscopic examination revealed characteristic changes of G. P. I. Large number of seizures before death.

*Cause of death.*—Exhaustion of seizures of general paralysis.

*Previous occupation.*—Laundress.

*Length of time in Asylum.*—Six years.

*Case 25.*—A. B., Claybury Asylum. 128.M./07. Male, age 30 years. Autopsy 28 hours after death.

*Weight of brain.*—1,275 grammes.

*Cause of death.*—Acute pulmonary tuberculosis.

*Previous occupation.*—Painter.

*Diagnosis of mental state.*—Melancholia. Probably congenital imbecile with epilepsy. Began to have epileptic fits at 14. Threatened to commit suicide.

*Length of time in Asylum.*—Five years six months.

*Case 26.*—I. E., Claybury Asylum. 7.F.07/2. Female, age 38. Autopsy 40 hours after death, body in cold chamber.

*Weight of brain.*—1,020 grammes. Some general wasting.

*Cause of death.*—Pulmonary tuberculosis.

*Diagnosis of mental state.*—Recurrent melancholia.

*Previous occupation.*—Hawker with her husband.

*Length of time in Asylum.*—Has been in asylum three times since 1894 Resident for eight years prior to death.

*Case 28.*—M. R., Horton Asylum. 4/1/08. Female, age 17 years. Autopsy five hours after death. (Brain sent to Claybury and placed in cold chamber. Forty hours elapsed before material was collected.)

*Weight of brain.*—1,075 grammes.

*Cause of death.*—Tubercular salpingitis. Tuberculosis of lungs, intestines and left elbow joint.

*Diagnosis of mental state.*—Dementia præcox (katatonic stupor).

*Previous occupation.*—General servant.

*Length of time in Asylum.*—Three months.

*Case 29.*—C. H., Bexley Asylum. 2/3/08. Male, age 23 years. Autopsy 26 hours after death.

*Weight of brain.*—1,165 grammes.

*Cause of death.*—Lobar pneumonia. No tubercle.

*Diagnosis of mental state.*—Dementia præcox.

*Previous occupation.*—Labourer. Reached only IVth Standard at age of 13.

*Length of time in Asylum.*—Four months, twenty days.

*Case 30.*—W. C., Rainhill Asylum, Liverpool. 2,471. M., June/07. Male, age 23 years. Autopsy 30 hours after death. Body not kept in cold chamber.

*Weight of brain.*—1,435 grammes. Some general wasting in pre-frontal region. Brain œdematous, considerable excess of fluid.

*Cause of death.*—Marasmus. No tubercle, lungs healthy.

*Diagnosis of mental state.*—Dementia præcox.

*Previous occupation.*—Packer in factory.

*Case 40.*—M. E. G., Claybury Asylum. 31.F./08. Female, age 47 years. Autopsy three hours after death.

*Weight of brain.*—955 grammes. R hemisphere 420 grammes, L hemisphere 360 grammes. The brain was extremely wasted and much congested, especially the L hemisphere, which was taken for analysis. Microscopical examination revealed the characteristic changes of general paralysis. Patient had seizures on and off since admission, more severe and frequent six months prior to death.

*Cause of death.*—Acute lobar pneumonia.

*Diagnosis of mental state.*—General paralysis of the insane.

*Previous occupation.*—Housewife. Married.

*Length of time in Asylum.*—Eight years.

*Case 41.*—M. D., Horton Asylum. 19/2/08. Female, age 28 years. Autopsy 18 hours after death.

*Cause of death.*—Tuberculosis of lungs and intestines.

*Weight of brain.*—1,095 grammes.

*Diagnosis of mental state.*—Dementia præcox.

*Previous occupation.*—Married. Housewife.

*Length of time in Asylum.*—Three years nine months.

*Case 42.*—C. O'C., Horton Asylum. 3/3/08. Female, age 27 years. Autopsy 13 hours after death.

*Weight of brain.*—1,190 grammes.

*Cause of death.*—Morbus cordis. Fatty degeneration. Bronchitis. No tubercle.

*Diagnosis of mental state.*—Dementia præcox.

*Previous occupation.*—Domestic servant. Single.

*Length of time in Asylum.*—Four years eleven months.

*Case 70.*—J. E. S., Department of Pathology, University of Chicago. 1/5/08. Male, age 43 years. Autopsy 4–5 hours after death.

*Weight of brain.*—1,400 grammes.

*Cause of death.*—Diffuse suppurative meningitis. (Duration of illness one week.)

*Mental state.*—Normal.

*Previous occupation.*—M.D. Surgeon.

### SUMMARY.

Methods have been devised and are given in detail (1) for the estimation of the proximate constituents of the brain, and (2) for the estimation of the elements phosphorus and sulphur in the various groups of constituents: protein, lipid, neutral and inorganic sulphur; protein, lipid

and extractive phosphorus. Preliminary analyses have been made in a few cases on the grey and white matter separately. On account of the small quantities of the elements actually present in the brain, these analyses have only been regarded as of value when the analysis of a 100-gramme uniform sample of the whole brain yielded confirmatory results.

The methods have been employed for the analysis of 20 brains from normal and pathological cases, with the following results:—

(1) Analysis of the brain at different ages shows that with the growth of the brain there is a decrease in the amount of moisture, proteins, extractives and ash; and the cerebrins, lipoids and cholesterin increase. Also there is an increase in the lipoid sulphur and phosphorus and a decrease in the neutral and inorganic sulphur and extractive phosphorus.

(2) Differences may be determined in the brains of different species, as demonstrated by a comparison of the human brain with that of the dog. This subject will be discussed by one of us (W. K.) at a later date.

(3) Comparison of brains from cases in which the causes of death were of an entirely different character showed no variations of importance. As the cause of death in one of these cases was "hæmorrhage due to erosion of peritonsillar abscess," it is apparent that the amount of blood present in the brain at death does not introduce any error of importance.

(4) Six brains from cases of dementia præcox have been examined, four by analysis of a uniform sample of the whole brain, and two by analysis of the grey and white matter separately. The results obtained on three cases already published (13) in which the analyses were made on the grey and white matter separately have been confirmed. Compared with the normal, the amount and distribution of phosphorus shows no marked change, but the neutral sulphur shows a great diminution while the inorganic and protein sulphur is relatively increased. Thus so far, nine cases in all have been examined and found to give results which, although varying among themselves, all tend in the same direction, *i.e.*, a *diminution of the neutral sulphur*. This variation appears to be independent of the cause of death and so far has not been found in other forms of insanity. It does not seem then unreasonable to suppose that the subjects of this mental disorder may possibly possess a general bodily inherent deficiency for oxidation processes. Examination of other tissues of the body for neutral sulphur and its proportion to the total sulphur contents would help materially to decide this point. In the meanwhile some support to this view of a general inherent bodily deficiency for oxidation processes is afforded by Pighini's observations on the increase of neutral sulphur in the urine in this disease.

(5) *Five brains* from cases of general paralysis have been examined, four by analysis of the brain as a whole and one by analysis of the white and grey matter separately. These cases were selected as controls of the analytical technique for the dementia præcox cases. *They do not show any marked change in the neutral sulphur content of the brain.* Compared with the normal, the results show that the destructive changes in this disease affect the brain generally and not one constituent in particular. There is, however, a tendency for the lipid phosphorus to be decreased, indicating a greater destruction of the phosphatids.

The application of these methods to the study of other tissues seems quite promising, and will be taken up in the course of time in the laboratories from which these observations have been reported.

In conclusion, we would express our indebtedness to the Pathological Sub-Committee of the London County Council for the many facilities afforded to us, and to Dr. F. W. Mott, F.R.S., for his valuable suggestions from time to time, and aid in obtaining the large amount of varied material, without which the work could not have been done.

Also we would thank the many gentlemen to whom we are indebted for normal and pathological material and clinical histories. Dr. Geo. A. Watson and Dr. Wells have kindly aided us with material and histological reports. Mr. H. C. Corper has assisted us with some of the phosphorous estimations.

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## **INTESTINAL OBSTRUCTION: AN OUTLINE FOR TREATMENT BASED UPON THE CAUSE OF DEATH.**

**A STUDY OF FOUR HUNDRED EXPERIMENTALLY PRODUCED LESIONS.<sup>1</sup>**

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PHYSIOLOGISTS often quote the sentiments of Montaigne: "With how little anxiety do we lose the consciousness of light and of ourselves." By this they would convey the idea that the act of dying is as painless as the act of falling asleep, and also as little perceived. Nevertheless, so strongly rooted in the human mind is the desire to live, that the profession of medicine has for its chief purpose the lengthening of human life. Hence, it is natural that the study of the causes which bring death to a cell, to an individual, or to a race of men has for long been a favorite and engrossing one. None of us without giving thought can realize how many and how different are the actual and underlying causes which terminate life. "The supreme fact," says Adami, "that sooner or later death comes to all men has profoundly affected all human thought, and the various religions of the world may be regarded as the evidence of man's determination to rise superior to the dissolution of his body." In the unicellular organism there is no destruction of the essential living matter but a multiplication thereof: each spore carries on the life. There is no death in the ordinary interpretation of the word. "It is with the appearance of the multicellular organisms that natural death enters into the world." In the cellular differentiation it comes about that the germ cells are given the inherent power of perpetual life, while the somatic cells, through the elaboration of which the individual organism reaches its final development—side-tracked groups, as it were—are destined to hold and live together for a span and then to undergo dissolution.

There are in general two forms of death which may befall the somatic cell—the physiological and the pathological. The pathological form of death may be induced by mechanical, physical, chemical, or bacterial agents. With these in this paper, which deals with the cause of death in duodenojejunal obstruction alone, I hope to demonstrate that we are not concerned. Physiological

<sup>1</sup> A research conducted under a grant from the Rockefeller Institute for Medical Research, and presented at a meeting of the Johns Hopkins Medical Society, Baltimore, March 1, 1909.

death, on the other hand, that form of dissolution of somatic entity which has been studied so thoroughly, so exhaustively, in the pioneer work from the Johns Hopkins laboratories, the death following parathyroidectomy and that associated with adrenal and pituitary destruction or removal, this physiological form of death is that which immediately concerns us. I understand physiological death to comprise not only the dissolution which follows the removal of certain substances by destruction of their secreting cells, such as the parathyroid, but also that dissolution of somatic life which is brought about by the mechanical interference with detoxication of the normal secretions of the body. Putting it concretely, I may say that a barrier which may prevent the normal distribution of enterokinase, or the normal reaction of anti-enzyme upon enzyme, is the cause of death which is physiological and somewhat of the same type as is the death brought about by removal of glandular secretions, so-called internal in character. It is not to be denied, of course, that these thoughts suggest ascribing to the duodenum a function, I believe, hitherto and as yet unknown, that of internal secretion.

In whatever manner this may be rightly considered, I ask you to allow me to start with the hypothesis that the intestinal barrier which closes the lumen works no ill to the organism save through an interference with the physiological exchange or balance of the duodenojejunal secretions, and that the resulting death is physiological in type. I ask you to assume for the moment—and for this we have adequate proof—that in intestinal obstructions there is no appreciable damage to the individual through the nervous system from the direct trauma of the obstruction; that there is no lethal infection of bacterial origin; that the element of decomposition of food does not enter into the picture at all—in short, that all pathological forms of death are eliminated. I shall strive to detail to you the simple, and withal possibly inefficient, measures which I have taken in my efforts to prove these things to myself.

**NERVOUS EFFECT OF INTESTINAL TRAUMA.** Clinically surgeons have recognized a difference of degree in the shock produced by cutting the intestine in various places: the nearer one approaches the pylorus, other factors being equal, the greater the shock. Indeed, in the execution of the well-known pyloroplasty operation devised by Finney, it has been, I believe, commonly recognized that a shock to the patient might occasionally be clinically noticeable at the moment of actual section of the pylorus. Operations which will be described later and in the course of which this highly sensitive mechanism was entirely removed or seriously injured, and furthermore operations in the remotely aboral portions of the small intestine which technically were associated with precisely the same amount of traumatism as those in the duodenum and jejunum, afford, under study, point blank evidence that the nervous shock or reaction (let it be called what it may) has nothing whatsoever to

do with the cause of death in the form of intestinal obstruction under consideration. Indeed, I have been led to the conviction that the infliction of even unusual mechanical traumas incident to various operative procedures upon the alimentary canal, not excepting the thoracic oesophagus, is incapable of producing more than a transient and unimportant nervous reaction. It stands to reason that if a dog would live for weeks with iliac obstruction and dies at the end of a few hours with duodenal obstruction, and that if the same dog can be made to live also for weeks, if but the slightest drainage be instituted, something far more important than mere mechanical injury is the cause of death. Obviously the traumatism in each case is equal. Incidentally, it may be noted that whatever nervous traumatism might be associated with the dilatation of the proximal loop, would presumably be greater where the greater dilatation occurred. In the majority of fatal cases of duodenal obstruction, there was little or no dilatation; it rarely has time to develop, whereas in the chronic aboral obstructions it is frequently found.

**INFECTION OF BACTERIAL ORIGIN.** No one could pretend to read the literature of intestinal obstruction and not be acquainted with the contributions which have been made from the Johns Hopkins Laboratory on the subject of bacterial emigration oral to the obstruction. These studies have contributed widely to our knowledge of the pathological type of death which unquestionably occurs from obstructions in the aboral portions of the ileum and from occlusions of the great gut. It is, however, the physiological form of death alone with which I am concerned; with the fulminating prairie-fire-like toxemias which, unheralded and often in a few hours, destroy the victims of duodenal obstruction. In dogs the clinical syndrome is usually typical; the muscular tremors localized in certain groups of muscles, the tachycardia, the rapidly advancing coma and death, followed by a characteristic stiffness. Clinically this picture is not far different from that observed after parathyroidectomy.

The pathological form of death which results from obstruction at or near the ileo-cecal valve and which may well be of bacterial or stercoraceous origin, has not been the special object of our study, and of it, therefore, I am not qualified to speak. I would note, however, in passing that it is slow and tortoise-like in overtaking the somites, suggesting in every detail a process quite different from that which has been the special subject of my studies. Furthermore, it should be noted that while the duodenum possesses a rich flora, it is exceedingly poor, at least in the healthy dog, in pathogenic forms, and continental observers, as well as your own, have found blood cultures taken from cases of duodenal obstruction entirely sterile.

With these premises then I ask you, for the academic purposes

of argument, to assume that in obstruction of the intestine two entirely separate and distinct forms of death are to be differentiated: the one duodenal and purely physiological, due to an unknown disturbance of the duodenal secretion or balance, the other ileo-colic and purely pathological; the one a true auto-intoxication, the other a true exo-intoxication.

**DUODENAL AUTO-INTOXICATION.** The following impressions have resulted from the study of over 400 cases of duodenal or orojejunal obstruction produced experimentally during the past five years at the Surgical Research Laboratory of Columbia University. Although the series is not small, the positive conclusions which are offered by it are regretfully small. This may be attributed in part to the natural deficiencies of the experimenter and to the great intrinsic difficulty of the task, and also very fairly in part to the fact that the work has been done at odd moments taken from an annoyingly engrossing practice and amid surroundings sadly incomplete and unfit. I mention this because I seek your indulgence.

A chronicle of the steps which have led to the above outlined position on intestinal obstruction is as follows. About five years ago, while modifying the McGraw elastic ligature so as to substitute common every-day twine for the perishable elastic, and also with a view to remove sizable apposed portions of gut and stomach wall, Dr. Weir at the time considering this a necessary part of the technique, we were suddenly confronted by the perplexing coincidence that all our animals died. There was no peritonitis; there was no gross lesion discoverable within or without the abdomen. We noted an early rigor mortis, and that before death the heart beat was accelerated, the gait was peculiar, owing to spasticity of the hind legs, and there were well-defined muscular twitchings. The technique had embraced the closure of the duodenum (Fig. 1). At that time no information was forthcoming as to the cause of these phenomena and I could find nothing regarding it in the literature. It was not long, however, before Roger, in France, began an allied series of experiments which to some extent corroborated our own findings. All these experiments, as well as the most recent ones, have been based on the utilization of this potential gastro-enterostomy by the twine triangular ligature as a control. It seems reasonable, and indeed is easily susceptible of proof, that the twine will cut out and give drainage in healthy stomachs of normal dogs at about a constant time, seventy-two hours. It is a coincidence of import that the physiological death which we have been studying usually takes place in about the same time as is necessary for stoma drainage to begin. Thus we have come to regard the cutting out of the stoma as a sort of index of resistance of the individual to the intoxication process, and we have proceeded to create different degrees of toxicity, which we found we could do by varying the position of the obstruction and of chronicling the results in terms

of gastric drainage. For it should be noted that almost any form of drainage, whether through the normal channel of the gut, or abnormally through the stomach or even through an entero-anastomosis of the aboral loop with the oral loop, will counteract the lethal effects of what we believe to be a physiological death, due to a disturbance of physiological intra-enteric enzymotic balance.

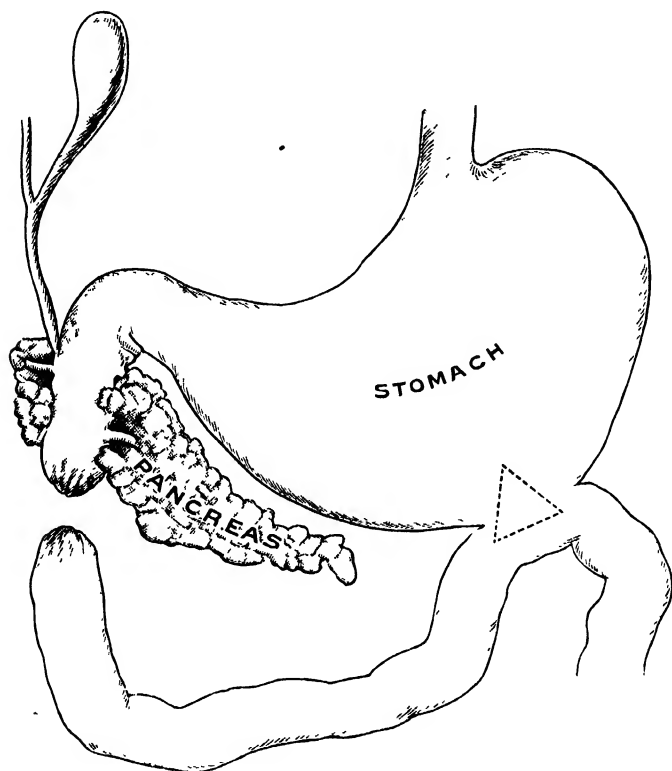


FIG. 1.—Duodenal obstruction; twine triangular control. In this class of experiments the bile and pancreatic fluid do not drain. Death before stomach drainage almost invariably occurs irrespective of the presence or absence of food in the stomach. This short-loop type should be given retrograde lavage or anti-enzyme serum from the long-loop class.

**DETERMINATION OF ABORAL LETHAL LINE FOR PHYSIOLOGICAL DEATH.** Logically the first step after determining by a long series of experiments that death followed the closure of the duodenum before drainage became established through the triangular stoma, was to ascertain the effect of practising a similar technique, but modified by placing the obstruction in the jejunum (Fig. 2). Another lengthy series of experiments determined, I believe with reasonable accuracy, that death would not occur in a medium-sized dog until

after the free drainage of the stomach, that is, several weeks, in any case when the obstruction lay more than 35 cm. aboral to the pylorus. This distance probably varies somewhat according to the size of the dog. The individuals exposed to this type of experiments will in future be referred to as the "long-loop dogs." For to them on account of the possible production of anti-enzymes, or protective

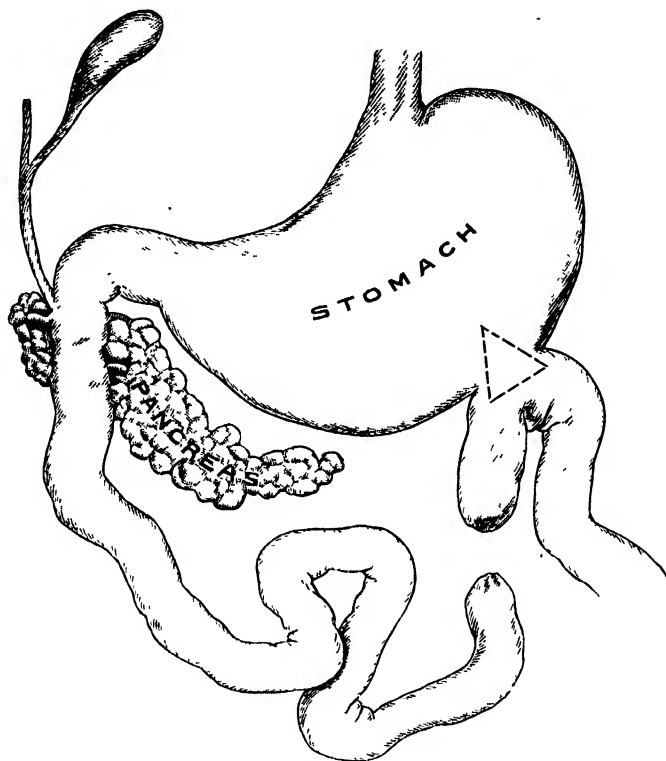


FIG. 2.—Jejunal obstruction. The length of the oral loop was more than 35 cm. In this class of experiments the dog lives until the twine cuts the stoma and often for months thereafter. Obstruction between this (35 cm.) and the greater pancreatic duct is almost always fatal before gastric drainage begins. Does the intestine detoxicate by the addition of an anti-enzyme, or does it save life by offering a larger field for absorption, thus diluting the toxic elements? This long-loop class may furnish a protective anti-enzyme serum for use in the short-loop class.

bodies of unknown character, there attaches a particular and perhaps a lasting interest. These long-loop dogs, barring ordinary accidents, survive the moderate toxemia to which the obstruction gives rise and will live often for several months without apparent discomfort or loss of body function. On autopsy, the oral segment was found to be but moderately dilated, as a rule, and the evenness

of digestive function suggested the phenomenon of gastro-proteid digestion. But the theoretical considerations which offer themselves as a result of this particular group of experiments must not interfere with this chronicle of plain observed facts.

**DETERMINATION OF ORAL LETHAL LINE FOR PHYSIOLOGICAL DEATH.** The next step naturally was to determine the oral limits of the point of obstruction beyond which one might go and not encounter physiological death within the time limit of triangular control. Because of the studies of Roger upon the gastrototoxic serum, it is of very great importance to know whether the lethal bodies take their origin from the gastric mucosa, from the pancreatic fluid, or from the duodenum. Roger states that simple ligation of the pylorus leads to this physiological death in from seventy-two hours to five days. That, it will be observed, is just without the limit of the twine triangular time control. It is true that Roger did not employ the delayed gastro-enterostomy for a control, and so much that is accidental is certain to enter into all this sort of work that one feels hesitancy either in commenting upon the results obtained by others or impressing one's own experimental findings. It is true, however, that we have observed this fact: Given free drainage of the duodenum through the normal channel, as shown in Fig. 3, the stomach may be obstructed at or near the pylorus more frequently without lethal result until the cutting through of the triangular stoma than if the obstruction were in the duct-bearing portion of the duodenum. Of course, in this type of experiment, with the cutting through of the stoma, there ceases at once to be a reason why the animals should not live for an indefinite period. I do not wish to be understood as saying that no physiological toxic elements arise from the stomach and that the source of the toxicity is strictly duodenal or pancreatic; but we have been struck by the fact that, discounting all consideration of biliary toxemia, which one seems quite justified in doing, at least the chief source of the poison is either in the duodenum or the pancreas. In the light of our present knowledge, and measuring the degree of toxicity by our time drainage unit, rather than attempting to measure it, as Roger has done, in terms of toxic units, we were led to assume that the source of the toxemia was not in the stomach but in the duodenum. This enabled us, for purposes of study, at any rate, to place the oral limit of the lethal line between the pylorus and the papilla of Vater, because until now the question of the influence of the bile has not yet been worked out.

The duct-bearing portion of the duodenum has long been recognized as an area having unknown and profoundly complex physiological properties, and it is in part the object of this paper to demonstrate that the most accurate interpretation and knowledge of the physiological processes going on in this short tube must form

the groundwork for future therapeutic progress, be it surgical or medical; not alone on the alimentary canal, but as one should logically expect, as well upon its great glands, the liver and the pancreas. The morphological relationship of the stomach, liver, and pancreas to the duodenum has been graphically portrayed by Mumford, who says that the pancreas, the liver and ducts, and the stomach hang like three apples on a single stem, the duodenum. Whatever affects one often affects the others. What more striking

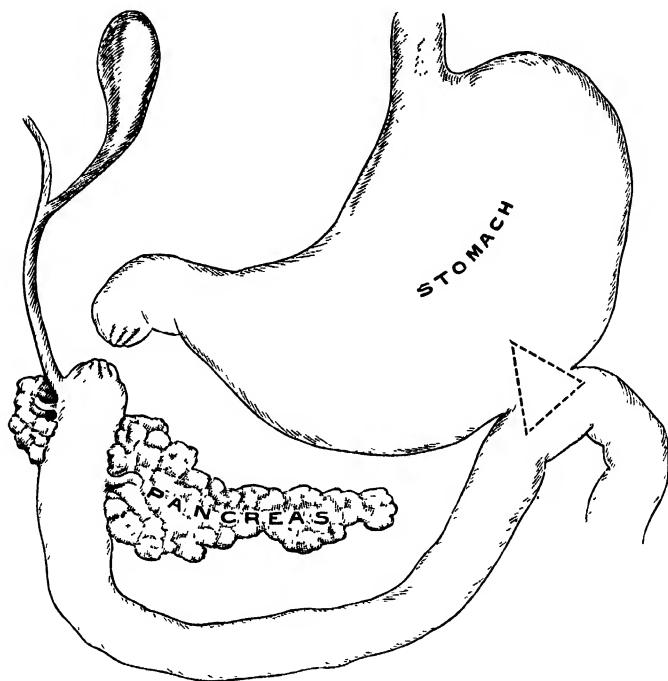


FIG. 3.—Duodenal obstruction; twine triangular control. Both the pancreatic fluid and the bile empty into the aboral loop. In this class of experiments there is usually no fatal outcome during stoma control, even if the stomach contains food at the time of the operation.

simile could one have? I submit that our studies have carried us still farther and that we are justified in considering not only the morphological relations, but the physiological relations as fixed. Is it not significant, in view of the close morphological relationship of these organs, that we have from unknown causes fatal hemorrhages into the pancreas, fatal dilatation of the stomach, fatal sequels to biliary operations which are not properly drained? I leave the discussion of this to those present more qualified to speak of it than I, and continue with the narration of our experimental observations.

**ELIMINATION OF THE BILE BY DUCT LIGATION; CHOLECYSTENTEROSTOMY AND TRANSPLANTATION OF THE POINT OF DISCHARGE INTO THE DUODENUM.** A long series of experiments, painful to the operator in point of technical detail, was created by which the bile was intended to drain into the ileum by the use of the ordinary suture opening between the gall bladder and the gut. For some reason, however, although the technical part was accurately executed, there

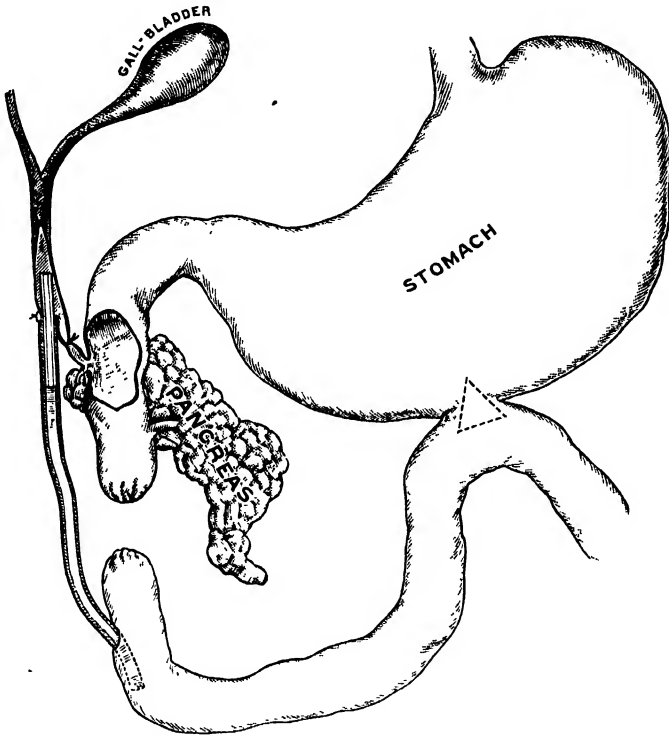


FIG. 4.—Extra-enteric transposition of the biliary secretion (Sullivan's technique). A glass cannula is inserted in the common bile duct; a rubber tube over the glass is stitched to the cut margin of the duct and introduced within the gut aboral to the intestinal obstruction. The bile duct is tied at the entrance to the duodenum.

was in every case a failure of the bile to drain. We finally concluded that there was either a change in its composition, owing to infection from the ileum, or that the secretory function of the liver cells had been abandoned or changed to an absorptive one. In any event, the bile draining or not draining, it was not possible for it to enter the oral loop, as the common duct had been ligated and divided, but the results, so far as the physiological death went, were the same. Death occurred in short-loop dogs before stoma-control

drainage. To fortify the position and to enable us to feel that we were not superimposing hepatic changes upon those under consideration, Mr. Arthur Sullivan, the senior laboratory assistant, devised means for the direct transplantation of the bile (Figs. 4 and 5). It comprises the reconstruction of the common bile duct, and will form the basis of a communication from him at a later date. The use of this simple and effective technique allowed of sufficient room between

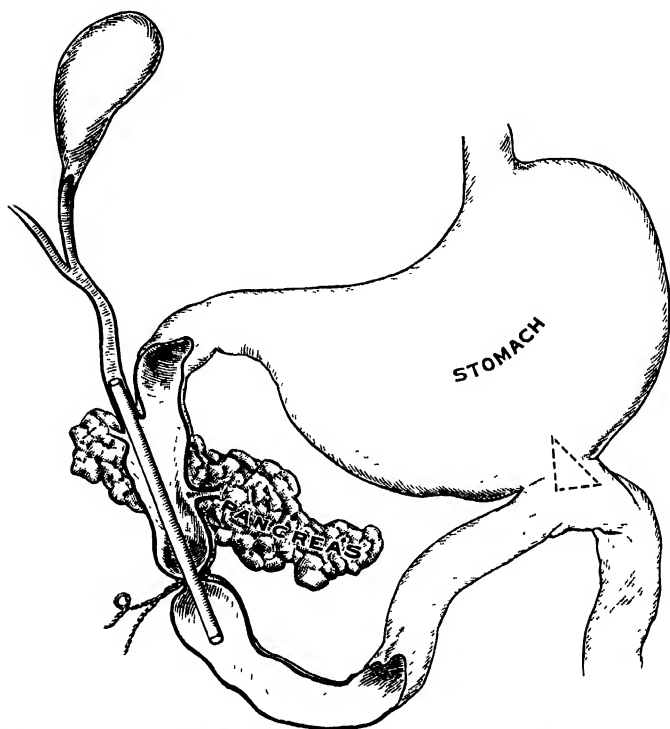


FIG. 5.—Intra-enteric transposition of the biliary secretion; twine stoma control. The forepart of the gut is shaved off to show the tube in position. This technique is difficult of execution and is further objectionable in not permitting actual section of the gut at the point of obstruction. There is apt to be leakage of the intestinal contents around the tube. This vitiates the experiment.

the transplanted biliary intake and the greater duct of the pancreas, either for complete section and invagination of the duodenum, or its ligation, as the case might be. Here, again, a sufficient series of experiments demonstrated that the supposed toxicity of the bile, at least in regard to obstructive death, was erroneous. It did not matter whether the bile emptied into the oral (Fig. 6) or aboral loop near the point of obstruction, whether the duct was simply ligated and cut, or whether cholecystileostomy was done, the lethal

outcome appeared rather conclusively still to be dependent entirely upon the position of the obstruction. In other words, bile, the salts of which are known to possess a very measurable degree of toxicity, was in no way connected with the physiological death under observation.

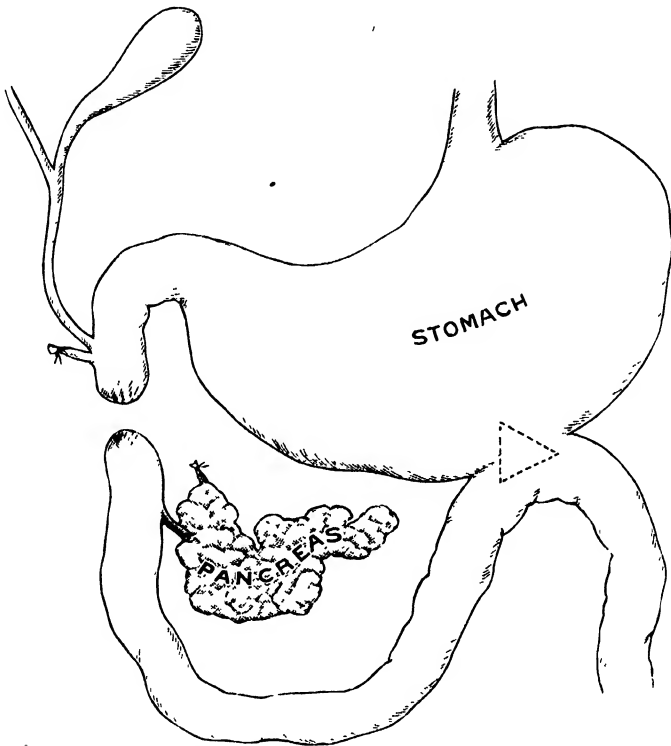


FIG. 6.—Duodenal obstruction between the pancreatic ducts; twine stoma control. The duct of Wirsung is tied and cut so that bile alone enters the oral loop. Drainage of the pancreas by the greater duct is adequate to prevent undue hardening or fat necrosis. The gland continues macroscopically in good condition. Dogs usually live during the period of stoma control

RELATION OF THE PANCREATIC SECRETION TO INTESTINAL OBSTRUCTIVE PHYSIOLOGICAL DEATH. The morphology of the parts does not allow a transposition of the pancreatic secretion, in the first place, because there are two ducts, and in the second place, because they are very short and firmly fixed to the pancreatic tissue (Fig. 7). Any interference with this, no matter however delicate, may lead to fat necrosis and other conditions which confuse the point of issue. By using Sullivan's tubes, however, we were able to move the point of entry of the bile as many centimeters aboral to the papilla of Vater as was convenient. After ligating the lesser

duct at its point of entrance beside the papilla and cutting it. we could make certain of having sufficient room to sever and invaginate the duodenum in such manner that all the pancreatic secretion might enter into the oral or the aboral loop. Opie has commented on the fact that drainage of the greater duct would usually suffice to prevent any undue hardening of the pancreatic gland because of retained secretion, and we have been glad to make use of his observation by tying and cutting the lesser duct. The result of these studies was conclusive in one regard: irrespective of the whereabouts of the discharged bile, the dogs lived during stoma control

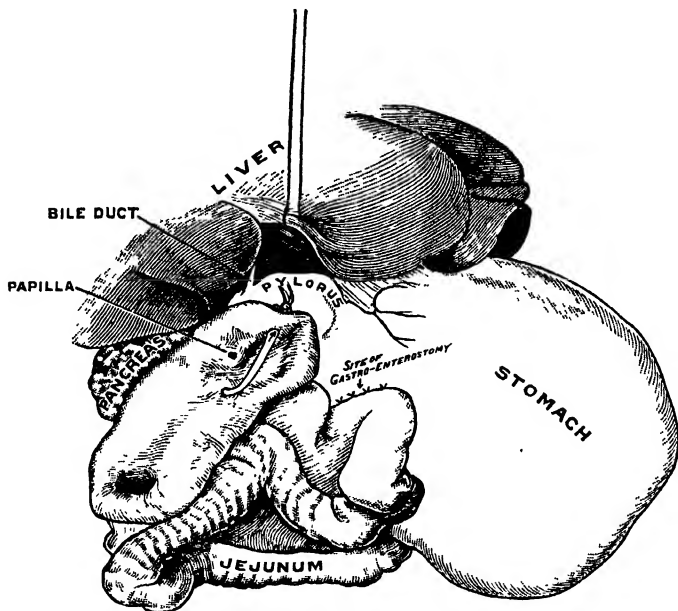


FIG. 7.—Attempted transposition of the pancreatic secretion oral to the duodenal obstruction produced by a ligature. The bile drains aborally. The lesser pancreatic duct is ligated.

when the pancreatic secretions drained aborally, and they died during stoma control when it was confined in the oral loop. I ask that this be not interpreted too positively, because enough conclusive work has not yet been done to justify too positive conclusions. This may well be pardoned, because of difficulties arising from the excessive sensitiveness of the duct-bearing portion of the duodenum, but we have gone far enough to justify our asking for corroborative evidence from others.

We understand that the secretions of the gastric mucosa may be toxic and that this toxicity may be increased many thousands of

times by an oral intestinal obstruction, but we have accomplished enough strongly to suggest at any rate that, whatever may be the nature and degree of this toxemia, a toxemia which develops from a disturbance in the duct-bearing portion of the duodenum contains poisons of a much graver nature. It is presumably not to be

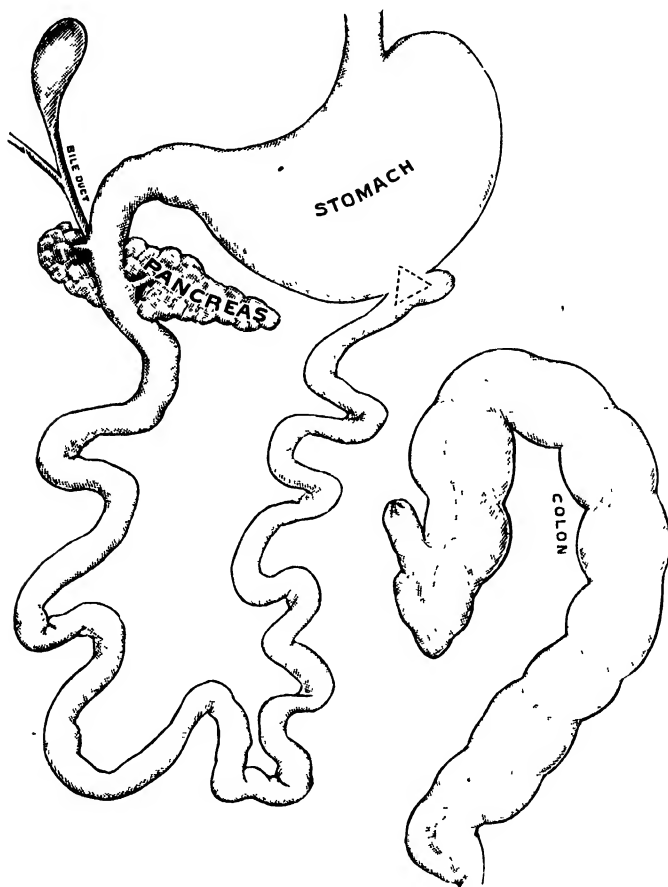


FIG. 8.—The entire small intestine forms the loop. The dogs lived several weeks.

denied that the increase in amount of gastrottoxins noticed after pyloric obstruction may well be brought about by the interference of a normal duodenal limitation of such toxin development. It is a point of perhaps more than academic interest that Weinland has found an antitryptic ferment to exist in the oral portion of the small intestine. Is one justified in supposing that, as the pancreatic

juice, grossly at least, appears to be the lethal agent, the danger from intestinal obstruction grows less and less the farther one places it from the pylorus, and therefore more and more in the antitryptic bearing portions of the canal, and this because of zymotic action. I do not wish to be understood as stating or even suggesting that trypsin alone or as such is responsible for the phenomena; no doubt there are many other ferments and antiferments occurring in the duct-bearing portion of the duodenum, but I speak of the

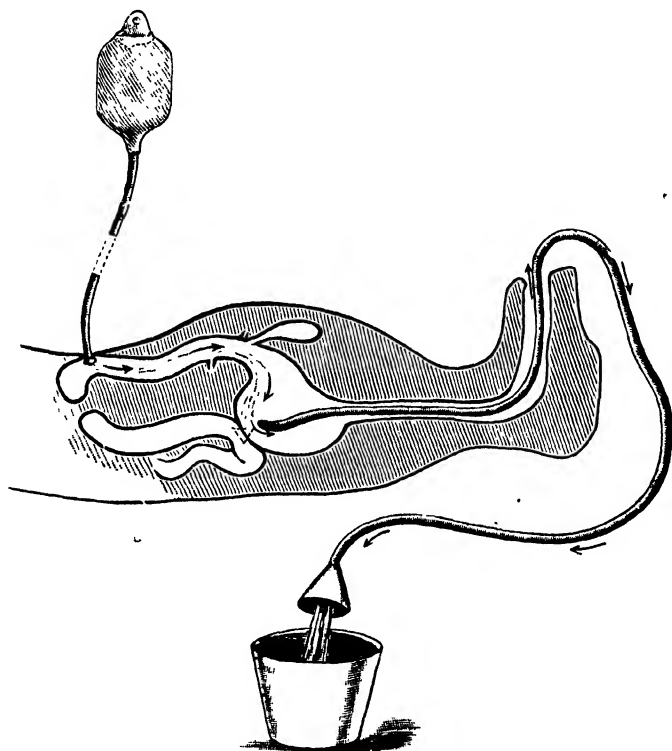


FIG. 9.—Retrograde lavage in a dog. This removes the toxic duodenal products quicker and more thoroughly than ordinary lavage.

trypsin simply as a representative of the class, because I understand its antibody to have been definitely found. It may indeed be that there is some form of internal secretion produced by the duodenum, possibly not directly associated with the processes of digestion, which is the fundamental factor in causing this physiological form of obstructive death. This should be capable of demonstration by experimental methods. Some such hypothesis is at present necessary in order to explain the singular phenomenon of the protective

power of the first 35 cm. of the intestine, the presence of which in the oral loop sufficing to prevent death before the opening of the triangular control. It may also explain the curious conditions shown in Fig. 8.

**THERAPEUTICS.** So far the suggestions as to treatment for these dogs have been based upon the belief that we had shown the region of maximum toxicity to be the duodenum rather than the stomach. It seemed, therefore, logical to irrigate the entire region rather than the stomach alone, and this by a sort of retrograde flushing (Fig. 9). This in a dog is easy to accomplish, the pyloric sphincter offering no obstruction either to alkaline or to acid irrigation. It is conceivable that some modification of this method might possibly be employed upon the human being, for constant irrigation of the duct-bearing portion of the duodenum would seem on experimental grounds to be the one logical method of preventing the absorption of the physiological poisons referred to.

A more philosophical method of meeting the conditions might be to transfuse the sick short-loop dog from a long-loop dog, which may be supposed to have manufactured, in the course of his temporary illness occurring before the establishment of gastric drainage, certain antibodies which might be protective to the short-loop dogs. We have done a little work in transfusing the long-loop into the short-loop dog, and have noted improvement in the latter; but this might come from accumulation of the fresh blood rather than from the presence of anti-enzymes. We are, therefore, endeavoring to obtain a serum from the long-loop dog, with which we hope to tide the short-loop dog over his period of hypertoxicity, so that he too may live until the triangular control gives gastric drainage and assures permanent life.



## THE COMPARATIVE TOXICITY OF THE CHLORIDES OF MAGNESIUM, CALCIUM, POTASSIUM AND SODIUM

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### INTRODUCTION

Studies on the action of inorganic substances upon living matter exist in abundance, nor is there any lack of researches having in view essentially the comparative toxicity of the substances under investigation. These researches, however, were frequently undertaken and carried on with a view to reducing the nature and cause of the toxicity of various substances to some chemical or physical principle. Such attempts at correlation between physiological action and some physical or chemical property of a substance were often made, especially soon after the proclamation of new facts or theories in physics and chemistry. These statements may be elucidated by the following few instances. James Blake,<sup>1</sup> stimulated by the electrolytic and atomic studies occurring at the early part of the last century, was led (1839) by experimentations (and speculations) to put forward the assumptions that physiological action emanates essentially from the electro-positive element and that within each isomorphic group of elements the toxicity grows with the weight of the atoms.

Richet,<sup>2</sup> whose experimental studies on fishes and the frog's heart brought forth evidence against the last-mentioned view of Blake (and partly also of Rabuteau), was himself led to connect the toxicity of substances with a physical factor: the toxicity of substances grows, he assumed, in inverse proportion to their solubility. Soon

<sup>1</sup> James Blake: Journ. of Physiology, v, p. 35, 124, 1884-5.

<sup>2</sup> Richet: Compt. rendu, T. 93, p. 649, T. 94, p. 742, T. 101, p. 707

after the discovery of the periodic system of the elements by Mendelejeff, Bodkin,<sup>3</sup> made an attempt to connect the toxicity of the elements with their position in that system. With the advent of the theory of dissociation of Arrhenius the toxicity of inorganic elements has been variously connected in some way or another with the electric charge of the ion. As an illustration we shall mention here the theory of A. P. Matthews.<sup>4</sup> According to this author the toxicity of inorganic substances depends upon the ease with which the electric charges of the ions are given up; the toxicity of the ions being therefore in inverse proportion to their solution tension and the toxicity of the salts being in inverse proportion to their decomposition tension. Matthews is also of the opinion that anions stimulate and cations depress the irritability of living tissues.

These diverging opinions have evidently, nevertheless, one common ground, and that is that the physiological action of a substance depends essentially upon the nature of that substance, the living tissue to be acted upon having very little or no voice in the matter. Apparently in harmony with this supposition are the methods employed in most of these investigations. The toxicity of the inorganic substances was tested upon lower animals, surviving organs, single tissues, etc., with the tacit assumption that that which was found for one tissue of one animal would hold true for another tissue of another animal, and what was true for an organ would hold true for a whole animal. The literature is full of instances of this kind. That the neglect of the character of the living matter as a factor in the toxicity of a substance would unavoidably lead to incongruencies, goes without saying. This may be illustrated by the experimental work carried out by Matthews in support of the theory that the solution tension is the essential factor determining the toxicity of a substance. Matthews has determined the toxicity of various inorganic salts by their action upon the eggs of the fish *Fundulus* and found that the toxicity of these salts grows as their solution tension decreases. In his table of toxicities we find, however, that sodium chloride is more toxic to the eggs than potassium chloride. While this fits in with

<sup>3</sup> Bodkin: *Centralbl. f.d. Med. Wiss.*, 1885, No. 48.

<sup>4</sup> A. P. Matthews: *Amer. Journ. of Physiol.*, x, p. 290, 1904.

his theory, inasmuch as the solution tension of sodium is lower than that of potassium, it is in complete disagreement with the facts as far as the tissues and organs of higher animals are concerned, for which, as everybody knows, potassium is incomparably more toxic than sodium.

The tendency to look at the physiological action exclusively from the angle of the chemical or physical structure of the toxic substance, obscures the right point of view. While it can be freely conceded that any and every physical or chemical property of a substance is capable of influencing the character and extent of its physiological action, it has to be borne in mind that the nature of the living matter to be acted upon is an important factor in the outcome of this action. A result obtained by the action of a substance on one tissue of an animal might not be the same as its action on another tissue of the same animal or on the same tissue of another animal. The effect of a substance upon an organ might be quite different from its effect on an entire animal. In short, in determining the toxic action of a substance the physiological composition of the living matter to be acted upon is at least as important as the physical and chemical nature of the acting substance.

In this series of experiments we sought to study the comparative toxicity of the four chlorides mentioned in the title of the paper in their action upon the entire animal. The action of the kations of these chlorides which are normal constituents of the animal body, have been in recent years the subject of extensive studies in general physiology. Their effects, however, were studied upon the lower animals or simple tissues. The study of the effect of these salts upon mammals is mostly carried on in pharmacological investigations. Here again we meet a predominant tendency to study the action of substances upon organs rather than upon entire animals and not infrequently the results obtained from the studies upon the former are applied without reserve to the latter. Furthermore, we here meet with the inclination to ascribe the toxic actions of the salts in question essentially to a physical property, termed "salt action"—meaning thereby the action due to osmosis. The potassium salts only are conceded to be capable of exerting a toxic effect due to some ion or chemical action. The great toxicity of magnesium which was recently

brought to light was interpreted by some writers as merely salt action. At any rate the prevailing view, based on studies on the heart, is that of the four salts, potassium chloride is the most poisonous. Incidental observations made us think that this view, as far as the entire animal is concerned, is erroneous. To clear up these and other questions, the present series of experiments was carried out.

#### THE EXPERIMENTAL FACTS

*Method.* The experiments were made on dogs. The salts were used in their molecular solutions and were introduced into the body by intravascular injections in four different ways: intravenously, through the jugular, the femoral and the splenic veins, and intra-arterially through the central end of a carotid artery. In the intravenous injections the solutions were running from a burette into the vein at the rate of about one to one and a half cubic centimeters per minute—except for sodium chloride, which had to be injected considerably faster, in order to finish one experiment in the course of one day. For the intra-arterial injections the outlet of the burette was connected with the artery by means of a capillary tube, while the top was connected with a large bottle in which the air pressure was raised to 280 mm. of mercury. The air pressure in the bottle was raised and easily maintained at a constant level, by connecting the bottle with a water faucet. The addition of small amounts of water at intervals kept the pressure practically constant. The solutions were then made to flow into the artery uniformly at the rate of about one cc. per minute, except again in the case of sodium chloride.

All the operations preparatory to the experiment were carried out under anesthesia—local or general, according to the requirements of each procedure.

The toxicity of a solution was judged solely by the size of the fatal dose. While during the course of the experiments the various effects of the injection of a solution were duly noted, the death of the animal was taken as the standard effect for a comparison of the toxicity of the solutions.

The amount of the molecular solution of each salt, per kilo body

weight, used up in bringing about the death of the animal, was taken as the toxic dose of that salt. The permanent stoppage of the heart was taken as the sure sign of death, although in some cases the respiration continued for a short time after the complete cessation of the heart beats.

For each of the four chlorides, twelve experiments were performed, that is, three experiments by each of the four above-mentioned methods of infusion. In many of the experiments blood pressure and respiration were studied by graphic methods. We shall, however, omit here the reproduction of any of the curves obtained, nor shall we enter upon detailed descriptions of these observations, as they will not contribute much to the further elucidation of our results, which in themselves are sufficiently clear.

*Magnesium Chloride.* In those experiments where an infusion was given through the splenic vein as well as in the intra-arterial injections, the animals had a preliminary subcutaneous injection of morphine (about 0.02 gm.) and were kept under ether narcosis during operation. In these cases the intravascular infusions were begun about 30 minutes after the operation was finished, to allow the animal to recover from the effects of the ether. In those experiments where infusion was made either through the jugular or the femoral veins, the slight operation of inserting a canula in the vein, was carried out under local anesthesia by cocaine, except in one case in which blood pressure was taken from the carotid artery and a bulb inserted between diaphragm and liver for transmission of respiratory movements; here both morphine and ether were employed.

The behavior of the dogs under the influence of magnesium chloride infusions was practically the same as described by Meltzer and Auer in experiments with intravenous infusions of this salt in rabbits. The animals were soon in deep anesthesia. In some cases the anesthesia was already complete while the respiration was still good and the lid reflex active. In all cases the respiration stopped before the heart. In some cases the heart continued beating for about three minutes after complete cessation of respiration. This survival of the heart was most pronounced in the experiments with intra-splenic injections.

In experiments such as these one does not expect to obtain exactly

uniform results. Besides the individual variations of the animals there is, in infusion experiments, the difficulty to maintain exactly the same rate of flow during the course of the entire experiment and also to have the same rate of flow in all experiments.<sup>5</sup> Nevertheless the average fatal dose for each of the three methods of intravenous injection was surprisingly constant. The average fatal dose per kilo body weight was for the jugular method 2.66 cc., for the femoral method 2.55 and for the splenic method 2.41 cc. of the molecular solution. The average fatal dose derived from all the three intravenous methods amounted to 2.54 cc. per kilo body weight. The average fatal dose as derived from the three intra-arterial injections was, however, perceptibly lower; it was 1.79 cc. per kilo body weight.

We append here three abbreviated protocols, one from each method of intravenous infusion with solutions of magnesium chloride.

*Experiment 1.* Infusion through the jugular vein. Male dog, 5900 gms. External jugular vein exposed and cannula inserted under local cocaine anesthesia.

2:25 p. m.—Injection began.

2:29—4 cc. in; heart 140, respiration 20 per minute.

2:34—9 cc. in; heart 160, respiration a little deeper and slower.

2:37—11.5 cc. in; heart same, respiration irregular, 13 per minute; some active expiration.

2:41—15 cc. in; heart 128, respiration 5 per minute, lid reflex not as active as before.

2:45—17 cc. in; respiration stopped, heart irregular.

2:47—18 cc. in; heart shows only a stray beat.

2:48—Heart beating regularly again, but slowly.

2:49—19 cc. in; heart stopped. No convulsions whatsoever.

*Experiment 2.* Infusion through the femoral vein. White female dog, 7050 gms. Vein exposed and cannula inserted under cocaine anesthesia.

10:15—Infusion began.

10:18—3 cc. in; vomiting.

10:23—8 cc. in; heart 128, respiration 38 per minute.

<sup>5</sup> The infusions into the splenic vein present a striking exception. In the experiments with magnesium, calcium and potassium salts the splenic infusions ran in uniformly at the rate of 1 cc. per minute.

10:31—15 cc. in; respiration 18 per minute, irregular, active expiration, lid reflex active.

10:33—17 cc. in; no respiration, heart irregular.

10:34—18 cc. in; heart stopped, a few strong beats afterward; animal died without convulsions.

*Experiment 3.* Infusion into splenic vein. Young male dog 13,750 gms. Morphine and ether anesthesia. Abdomen opened transversely, bleeding controlled; cannula in a splenic vein; viscera continually kept covered with towels saturated with warm saline.

10:59—Started infusion of magnesium chloride in  $\frac{M}{7}$  solution.

11:13—14 cc. in; heart 114, respiration 56 per minute.

11:20—21 cc. in; heart same, respiration 46, slightly active expiration.

11:23—Lid reflex slightly reduced, severe pinching of lips with mouse-tooth forceps not felt.

11:32—33 cc. in; heart rate practically same, respiration 12 per minute, very feeble.

11:39—40 cc. in; respiration stopped.

11:40—41 cc. in; heart still beats occasionally.

11:41—42 cc.; heart almost stopped. Infusion discontinued.

11:42—Heart stopped.<sup>a</sup>

In the intra-arterial experiments, respiration and blood pressure were studied by the graphic method. Neither tracings nor protocols of these experiments will be given here.

*Calcium Chloride.* The mode of anesthesia was the same as in the experiments with magnesium chloride, except that sometimes ether was lightly given during the infusion. Without anesthesia the animal was apparently awake during the infusion of the calcium solution, and until shortly before death, sensations and lid reflex remained practically unchanged. The rate of respiration during the course of the infusion was irregular, but in general, it became first increased and then decreased again. The character of the respiration, however, was typical for the calcium infusion. With the progress of the experiment inspiration gradually became labored, the accessory muscles

<sup>a</sup> It should be mentioned that the urine of most of these magnesium animals contained sugar after death even when they received no morphine. There were, however, a few animals whose urine contained no sugar.

being called into play, and the end of inspiration often showed a tetanic pause. Expiration also became active and apparently had often to overcome closure of the glottis. The heart rate was also irregular, but as a rule was at first decreased to become later considerably increased. With the progress of the infusion the rhythm became irregular, the respirations appearing often in groups and changing pronouncedly with the phase of respiration. The fatal termination was as a rule introduced by convulsions, the heart in many cases stopping before the respiration.

In the infusion of the calcium chloride the fatal dose shows a greater individual variation than in infusions with the other salts. The labored respiration influenced greatly the rate of flow. The infusions through the femoral vein contain one experiment with an exceptionally high fatal dose—11 cc. per kilo body weight—which is completely out of proportion to the figures obtained in the other experiments. This exceptional case makes the average fatal dose for the femoral infusions nearly twice as large as the average fatal dose for the jugular infusions. The average fatal doses for the four methods of infusion were as follows:—jugular, 3.02 cc., femoral 5.32, splenic 4.83 and carotid 2.81. Here again the intra-arterial method of infusion proved to be slightly more fatal than any of the other methods.<sup>7</sup>

The following abbreviated protocols illustrate the experiments with calcium infusions:

*Experiment 4.* Infusion through the jugular vein. Female dog, 4700 gms. Jugular vein exposed and cannula inserted under local (cocaine) anesthesia.

9:58—Started infusion.

10:05—7 cc. in; labored respiration with marked inspiratory pause, 16 per minute, heart 170.

10:08—Spasmodic closure of the glottis seems to interfere with expiration.

10:10—12 cc. in; respiration irregular, lid reflex active; animal struggles some.

10:20—22 cc. in; heart 72, respiration 8 per minute.

<sup>7</sup> In a few of the calcium infusions the urine contained a reducing substance.

10:24—26 cc. in; heart stopped, lid reflex almost gone, animal breathing irregularly but quietly.

10:26—Respiration stopped.

*Experiment 5.* Infusion through femoral vein. Male dog, 7900 gms. Vein exposed and cannula inserted under local anesthesia.

9:51—Infusion began.

10:01—10 cc. in; heart 138, respiration 36 per minute.

10:04—12 cc. in; convulsion, heart stopped. Respiration continued for nearly three minutes longer.

*Experiment 6.* Infusion through the splenic vein. Male dog, 9250 gms. Anesthesia and preparation as in the magnesium infusion. Before beginning infusion, heart 104, respiration 20 per minute.

10:40—Started infusion.

10:50—10 cc. in; heart 64, respiration 24 per minute.

11:10—27 cc. in; heart 176, respiration 32, lid reflex active, skin sensitive, whines slightly with each expiration.

11:25—45 cc. in; heart 176, very weak, respiration 8 per minute, labored, with long inspiratory pauses, lid reflex very active, skin sensitive.

11:30—50 cc. in; no femoral pulse, heart shows fibrillary contractions, lid reflex gone, sensibility gone, but respiration continues.

11:32—Still some motion of the heart and some respiration.

11:33—53 cc. in; heart and respiration stopped.

*Potassium Chloride.* The anesthesia which was employed in this series of experiments was in general the same as that employed in the calcium experiments. That is, besides the anesthesia employed during the operation, ether had to be given sometimes during the course of the infusion, especially during the splenic and intra-arterial infusions. Otherwise the animal was wide awake during the infusion, apparently even more so than in the calcium experiments. The heart rate was usually first increased and then gradually slowed down until it stopped. The rate of respiration did not have a regular course; in some cases it increased considerably during the infusion to slow down rapidly only shortly before death; in other cases it slowed down at the very beginning of the infusion or soon after, and in other cases again it increased and decreased at various times in the course of the infusion.

The lid reflex remained very active until death. There was a great deal of salivation during the infusion of potassium chloride and also frequent swallowing. Vomiting occurred in a few cases. With one exception there were no convulsions.

The order of stoppage of the heart and respiration did not follow a fast rule. In some cases the respiration stopped a trifle before the heart, in others the heart stopped shortly before respiration.

In the experiments in which the solution of potassium chloride was given intra-arterially, the course was somewhat different from the one observed in the intravenous injections. In the intra-arterial experiments blood pressure was taken (from the femoral artery) and a graphic record of the respiration was obtained from a rubber balloon inserted between the diaphragm and liver. In all such experiments the blood pressure generally remained high and the heart rate rapid until after a fairly large quantity of the solution was infused, when the blood pressure sank and the heart rate slowed down until both failed together. In other words heart rate and blood pressure remained fairly high until shortly before death. The respiration, however, became affected fairly soon after the beginning of the infusion. At short intervals there would be spells of very rapid breathing with strong active expirations. (In connection with these spells there would be temporary falls of blood pressure of moderate extent.) The spells would be at first of short duration, ten to twenty seconds, and occurring every two or three minutes. Toward the end, however, they lasted longer and were more frequent.

The average fatal dose of the potassium salt for each of the four methods of injection was as follows: jugular, 5.48 cc.; femoral, 4.37 cc.; splenic, 6.29 cc.; carotid, 8.45 cc. per kilo body weight.

There is a noteworthy difference between the potassium salt and the salts of magnesium and calcium, in the relation of the fatal dose of the intra-arterial to that of the intravenous method of injection. While for the two last-named salts the fatal dose by the intra-arterial method was smaller than by any of the three intravenous methods, for the potassium salt the dose by the intra-arterial method was larger than by any of the intravenous methods. We shall discuss later the reason for this contrast. A few illustrating protocols follow:

*Experiment 7.* Infusion through jugular vein. Female dog, 6000 gms. Vein exposed and cannula inserted under local anesthesia. Heart 160, respiration 44 per minute.

10:05—Infusion began.

10:16—11 cc. in; heart 172, respiration 24 per minute.

10:25—20 cc. in; heart very fast during inspiration and slow during expiration, respiration 23 per minute.

10:32—27 cc. in; heart 88, quite irregular, respiration 29. Salivation. Notices objects perfectly.

10:39—34 cc. in; heart 64 per minute, very weak.

10:40—No femoral pulse, lid reflex gone.

10:41—37 cc. in; heart still beating, respiration gone.

10:42—39 cc. in; heart stopped.

*Experiment 8.* Infusion through the femoral vein. Female dog, 8300 gms. Same preparation, as in previous experiment. Heart 104, respiration 30 per minute.

2:21—Started infusion.

2:28—7 cc. in; heart 132, respiration 76 per minute, shallow; salivation.

2:51—30 cc. in; heart about the same, respiration 180 per minute; marked salivation.

2:57—36 cc. in; heart 40 per minute, irregular, respiration still rapid. Lid reflex normal; animal perfectly conscious.

2:59—38 cc. in; heart 16 per minute.

3:02—40 cc. in; heart stopped for a short time, then started again.

3:04—Respiration stopped.

3:05—42 cc. in; heart stopped. No convulsions.

*Experiment 9.* Infusion by the splenic vein. Male dog, 7650 gms. Morphia-ether anesthesia. Heart 136. Respiration 36 per minute.

10:20—Infusion began.

10:40—20 cc. in; heart 120, respiration 22.

10:50—30 cc. in; heart and respiration a trifle slower, lid reflex active; animal perfectly quiet.

11:13—53 cc. in; heart 48; respiration 12 p. m., lid reflex active, sensation normal.

11:16—Heart very slow. Vomiting.

11:25—Lid reflex much reduced; heart almost stopped; repeated vomiting.

11:26—65 cc. in; heart stopped; lid reflex gone; still some respiration.

11:30—Respiration stopped.

*Sodium Chloride.* On account of the very long time which the animals in these experiments had to stay on the holder, all received a subcutaneous injection of morphine besides the usual anesthetic procedures. As stated before, the rate of flow in these experiments was sometimes more than twice as fast as in the experiments with the other salts. As it was, it took sometimes six to seven hours and longer to reach finally a fatal dose. One animal, which was taken off the board after it received about 28 cc. per kilo body weight, survived the procedure without any sign of unfavorable symptoms. The average fatal dose for all four methods of infusion amounts to about 63 cc. per kilo body weight. Before the infusion reached about 300 cc. of the solution, there were hardly any noteworthy symptoms except perhaps that in some cases the respiration became quite slow. When the infusion approached the end of the fourth hundred, for dogs of 7 or 8 kgms., muscular twitchings began which gradually developed into strong clonic convulsions, in which practically all muscles were involved. The convulsions, however, never terminated directly in the death of the animal; without any exception there was an interval lasting from ten to twenty-three minutes between the end of the convulsion and death. During this interval respiration and pulse were sometimes still favorable, reflexes active, and sensation present. Gradually all began to fail, the respiration as a rule stopping at least two or three minutes before the heart. During the entire course of the experiment the heart suffered little. Sometimes there was a moderate increase, sometimes a decrease in the rate. The rate of respiration, however, became gradually perceptibly diminished, a result which could not be ascribed to the small amount of morphine used. After the convulsions ceased, in some cases respiration and pulse went up again for a short time. The rectal temperature after the convulsions was often 45° C. in spite of the great loss of heat due to being stretched on the board for seven or eight hours. Diuresis set in very early and in most cases there was continuous dribbling from the urethra. In one case the collected urine was twice the quantity of the injected

salt solution.<sup>8</sup> In only one case did fluid defecation occur during the infusion. Salivation was present, but not excessive.

The fatal dose per kilo body weight for each of the four modes of injection was as follows: jugular vein, 64.67 cc.; femoral vein, 46.12 cc.; splenic vein, 77.56 cc.; carotid artery, 64.47 cc. The fatal dose for the femoral method has to be eliminated, since in two of the dogs the experiment was terminated before the fatal dose was reached. In considering the average fatal dose for sodium chloride it has to be kept in mind that the rate of flow for this salt was much greater than for the other salts. If the same rate had been used for the other salts, their fatal doses would probably have been reduced to one-half or still less.

The following are a few very abbreviated protocols of the sodium chloride experiments:

*Experiment 10.* Infusion through the jugular vein. Male dog, 8300 gms. Vein exposed and cannula inserted under local anesthesia, then 0.2 gm. morphine injected subcutaneously.

9:56—Infusion began. Heart 88, respiration 12 per minute.

10:51—60 cc. in; heart 60, respiration 60 per minute.

11:51—120 cc. in; heart 56, respiration 86, urine contains no sugar.

1:40—210 cc. in; heart 44, respiration 16, lid reflex active.

2:15—270 cc. in; heart 44, respiration 10 per minute.

3:35—360 cc. in; heart 44, respiration 13, perfectly quiet.

4:04—420 cc. in; heart 56, respiration 12, lid reflex active.

4:20—450 cc. in; very slight muscular twitching.

5:00—540 cc. in; twitching increased, some hyperesthesia, heart 104, respiration 9 per minute, lid reflex good.

5:30—600 cc. in; convulsive twitching quite prominent, marked hyperesthesia, has already secreted 1200 cc. of urine.

5:45—630 cc. in; strong convulsions.

6:00—660 cc. in; convulsions not so intense, heart 142, respiration 16 per minute, lid reflex a little sluggish.

6:09—690 cc. in; quiet now.

<sup>8</sup> It is a noteworthy fact that in spite of the large amount of concentrated solution of sodium chloride injected intravascularly, the urine contained no sugar. Even in the case in which sugar appeared after the injection of morphin, no sugar could be found in later stages.

6:15—711 cc. in; respiration stopped, lid reflex gone, heart still beating.

6:20—715 cc. in; heart stopped.

*Experiment 11.* Infusion through the femoral vein. Female dog, 6550 gms., preparation as before.

11:45—Injection began, heart 120, respiration 18 per minute.

12:39—60 cc. in; heart 88, respiration 16 per minute.

3:05 —210 cc. in; heart 56, respiration 18 per minute, voids every few minutes a clear urine, lid reflex active, sensation normal, perfectly conscious but quiet.

3:31—240 cc. in; heart 68, respiration 11 per minute, some quivering of muscles.

4:25—300 cc. in; muscular twitching quite noticeable, continual dribbling from urethra.

4:55—Very strong convulsions, heart rapid, respiration labored (36 per minute), lid reflex active, perfectly conscious.

5:10—Convulsions ceased.

5:18—390 cc. in; respiration ceased, lid reflex gone, heart still beating regularly.

5:21—393 cc. in; heart stopped.

*Experiment 12.* Infusion through splenic vein. Female dog, 8200 gms. Morphine-ether anesthesia.

10:20—Infusion began. Heart 96, respiration 14.

11:05—90 cc. in; heart 104, respiration 14.

11:50—180 cc. in; heart 128, respiration 12 per minute, quiet.

12:33—270 cc. in; heart 168, respiration 16 per minute, lost 35 cc. blood from a small splenic vein.

1:45—390 cc. in; heart 184, respiration 12 per minute, lid reflex active, increasing muscular twitching, some hyperesthesia.

2:28—480 cc. in; marked convulsions.

2:38—510 cc. in; very strong convulsions.

3:04—Convulsions gone, lid reflex active, sensitive to pain, but perfectly quiet, respiration 28 per minute, easy and regular; heart 76 per minute, weak but fairly regular. Blood pressure low.

3:06—560 cc. in; respiration stopped quietly, heart still beating; lid reflex present, but sluggish.

3:08—Lid reflex gone, no sensation, but heart still beating.

3:10—570 cc. in; heart stopped.

The following tables contain the fatal doses of every experiment with the four chlorides (reduced to per kilo body weight and expressed in cc. of the molecular solutions). They contain also the average fatal dose for each of the methods of injection expressed in grammes weight of the dry salt:

TABLE I.  
*MgCl<sub>2</sub> (+ 6 H<sub>2</sub>O).*

METHOD OF INJECTION.	NUMBER OF EXPERIMENT.	FATAL DOSE PER KILO OF BODY WEIGHT.	
		M Of $\frac{1}{1}$ Sol. in cc.	Average of anhydrous salt or each method in grams.
Jugular vein. . . . .	5	1.57	.253
	16	3.20	
	23	3.22	
Femoral vein. . . . .	1	3.17	.243
	21	1.93	
	22	2.57	
Splenic vein. . . . .	32	2.91	.229
	37	2.50	
	38	1.83	
Carotid artery. . . . .	4	1.54	.149
	10	1.83	
	11	2.02	

In Table II the third experiment by the femoral method presents, as stated before, such an exception that it ought to be excluded from calculation. The average for this method in dry salt would then amount to 3.04 grms., which approaches the one obtained by the jugular method.

In Table IV, in the first two experiments by the femoral method, Nos. 4 and 46, the infusion was discontinued before the animals died. These two experiments therefore have to be eliminated. The fatal dose of the remaining single experiment expressed in grammes of the anhydrous salt amounts to 3.51, a figure again approaching that obtained by the jugular method.

TABLE II.  
 $\text{CaCl}_2 (+ 6 \text{H}_2\text{O})$ .

METHOD OF INJECTION.	NUMBER OF EXPERIMENT.	FATAL DOSE PER KILO OF BODY WEIGHT.	
		Of $\frac{\text{M}}{1}$ solution in cc.	Average, for each method, of anhydrous salt in grams.
Jugular vein .....	7	1.78	.335
	26	5.53	
	27	1.75	
Femoral vein .....	3	3.18	.590
	25	1.83 3.04	
	29	10.96	
Splenic vein.....	40	5.75	.536
	41	3.71	
	43	5.04	
Carotid artery.....	18	1.94	.312
	30	1.44	
	31	5.06	

TABLE III.

 $\text{KCl}$ .

METHOD OF INJECTION.	NUMBER OF EXPERIMENT.	FATAL DOSE PER KILO OF BODY WEIGHT	
		Of $\frac{\text{M}}{1}$ solution in cc.	Average, for each method, of anhydrous salt in grams.
Jugular vein .....	6	4.86	.603
	35	6.16	
	36	5.44	
Femoral vein.....	2	5.06	.520
	33	4.20	
	34	4.93	
Splenic vein.....	39	6.87	.692
	44	3.45	
	45	8.55	
Carotid artery.....	20	7.24	.930
	28	8.50	
	42	9.63	

TABLE IV.

*NaCl.*

METHOD OF INJECTION.	NUMBER OF EXPERIMENT.	FATAL DOSE PER KILO OF BODY WEIGHT	
		M of $\frac{1}{2}$ -sol. in cc.	Average, for each method, of anhydrous salt in grams.
Jugular vein . . . . .	8	38.88	3.78
	48	86.14	
	49	68.70	
Femoral vein . . . . .	4	27.77	2.70
	46	60.61	
	47	60.00 . . . . .	
Splenic vein . . . . .	51	69.51	4.54
	52	69.86	
	55	93.33	
Carotid artery . . . . .	50	48.10	3.79
	53	82.97	
	56	63.16	

## DISCUSSION

If we take an average of the fatal doses per kilo animal by all four methods of injection, the fatal dose for each of the four chlorides, expressed in grammes weight of the anhydrous salt, is as follows:

MgCl<sub>2</sub>—0.223 grm.; CaCl<sub>2</sub>—0.444 grm.; KCl—0.464 grm. and NaCl—3.70 grms.

From these figures we learn in the first place that magnesium is the most and sodium the least poisonous of the chlorides. According to these figures potassium and calcium seem to be of equal toxicity. However, these figures do not yet express the exact conditions. Since the acid radical or the anion is in all four salts the same, any difference in the toxicity must be due to a difference of their basic radicals or kations. The comparison, therefore, must be based not upon the weight of the salts, but upon the weight of the kation elements contained in the fatal doses of each salt. On this basis the fatal doses of the four chlorides, as they were derived from our experiments, give us the following figures for the toxicity of the four kations under discussion:

Mg—0.057; Ca—0.160; K—0.243; Na—1.456.

From which we learn that calcium is more toxic than potassium.

Leaving for the present the question of toxicity of sodium out of discussion, our experiments show that in the order of toxicity magnesium occupies the highest position, with calcium second, while potassium is the least toxic of the three elements. This result does not seem to be in accord with the prevailing view. From a review of the literature one gains the impression that potassium is considered as the most toxic of the inorganic elements of the animal body. There are numerous studies on the toxicity of the chloride of potassium with only a very few studies devoted to the toxicity of calcium or magnesium. This view of the greater toxicity of potassium is directly supported by figures furnished by Binet<sup>9</sup>. In a table on the toxicity of various inorganic elements in which the toxicity of strontium is set as 1, the toxicity of magnesium is given as 2.5, that of calcium as 3.0, and that of potassium as 7.0; in other words, potassium is nearly three times as toxic as magnesium, while calcium is only slightly more toxic than the latter. Without entering into a discussion of the merits of the statement of Binet we shall only point out that they were derived from experiments made with subcutaneous injections in frogs, while our experiments were made, as detailed above, by intravenous injections in dogs. When we remember that the fatal action of magnesium upon mammals is essentially through its effect upon the respiratory center, we may concede that the toxicity of magnesium loses its rank in its action upon the frog, since this animal manages to continue to live for some time without the respiratory center and solely by the aid of cutaneous respiration. As to mammals we may say this: while our experiments entitle us to apply our results strictly only to dogs, our casual observations on other mammals led us to surmise that these results will hold good also for other members of this class, especially with regard to the greatest toxicity of magnesium.

With regard to the toxic effects of the chlorides of magnesium, calcium and potassium, there can be no doubt that they are chemical in character and not physical, that is, that the fatal results were

<sup>9</sup> Binet: *Compte rendu*, T. 115, p. 251.

not caused by the osmotic effect of the hypertonic solutions. The simple fact that the fatal doses of the solutions of these three salts form only a very small fraction of the fatal dose of the solution of sodium chloride, is sufficient evidence that osmosis had very little share, if any, in the fatal effect produced by these salts. All four salts were given in their molecular solutions, and the slight differences in the dissociability of these salts in these concentrations, surely cannot account for the great difference in the toxicity of the solutions of chloride of sodium and the other three chlorides.

As to the nature of the toxic effect of the sodium chloride solutions in our experiments we have no direct experimental proof against a claim that it was exclusively or essentially due to osmosis. The phenomena attending the death of these animals were those usually described as salt action, that is, a desiccating effect upon the vital tissues. That such a withdrawal of water actually took place in these experiments was clearly demonstrated by the fact that in some of the experiments the quantity of urine voided by the animal during the infusion was twice as much as the quantity of infused solution of the sodium chloride. However, while we have to admit that in the toxic phenomena produced by sodium chloride the physical factor of osmosis may have a large share, it seems to us that we should not lose sight of the chemical action of the sodium ion as an important factor in the toxicity of sodium chloride solution. The twitchings and convulsions which are constant symptoms in these infusions and which are ascribed by many pharmacologists to the drying of nerve or muscle tissues, may well be compared to the twitchings of frog muscles when immersed in pure solutions of sodium chloride, as observed and studied by Ringer, J. Loeb, and others, and which surely are not due simply to osmosis. The fact that in all our experiments the convulsions subsided before death, during which interval spontaneous respirations, reflexes and even a degree of consciousness persisted, seems to speak in favor of the comparison with an immersed muscle whose twitchings subside before the loss of irritability. Twitching due to drying should subside only with the loss of irritability. That sodium chloride may have a distinct toxic effect of a chemical nature was well proven by the observations of Loeb<sup>10</sup>

<sup>10</sup> J. Loeb: *Amer. Journ. of Physiol.*, iii, 327, 1904.

upon fundulus eggs. In fact, we shall refer to the extensive writings of this investigator for a further discussion of this subject. We shall only state here summarily our belief that the phenomena observed by us in the experiments with the infusion of sodium chloride had their origin partly in the toxic action of the sodium ion, and partly in the osmotic action of the hypertonic solution. A noteworthy point in the fatal terminations of the dogs from infusion of sodium chloride is the fact noted by us that in all experiments the respiration subsided several minutes before the heart stopped and without any terminal convulsions. From the observations of Locke,<sup>11</sup> Cushing<sup>12</sup> and others, we know now that by perfusion of the posterior extremities with sodium chloride the indirect irritability disappears long before the direct; in other words, sodium chloride exerts upon the motor nerve endings a "curare-like" action. May not the early stoppage of respiration in these experiments without accompanying convulsions have been due to such a "curare-like" action of the sodium chloride upon the motor nerve endings?

The early death of the animals in the infusion experiments with magnesium chloride was invariably due to a cessation of respiration; it was not accompanied by any convulsions. Meltzer and Auer<sup>13</sup> assume that death is due to a paralysis of the respiratory center. In this connection the fact is interesting that for the magnesium salt the method of injection through the carotid artery gave the smallest fatal doses; that is, by injecting through the artery, the animals died sooner than from intravenous injections. The explanation seems to be this: By injection through the veins the magnesium solution is first thoroughly mixed with the blood within the heart before it is evenly distributed to all parts of the body. By injection through the central end of the carotid artery the magnesium solution soon after reaching the aortic arch turns through the vertebral arteries toward the medulla oblongata, which thus receives a greater share and a greater concentration of magnesium than it would receive by the intravenous injection.

Another interesting phenomenon which was observed in the experi-

<sup>11</sup> Locke: *Centralbl. f. Physiol.*, viii, p. 166, 1894.

<sup>12</sup> Cushing: *Amer. Journ. of Physiol.*, vi, p. 77, 1902.

<sup>13</sup> See Meltzer and Auer: *Amer. Journ. of Physiol.*, xxiii, p. 140, 1908.

ments with potassium chloride can be viewed in the very same light. Here we saw that experiments with the intra-arterial injections furnished larger fatal doses than the intravenous injections,—that is, the injections through the carotid artery proved less dangerous than the intravenous injections. The essential cause of death from potassium chloride is cardiac paralysis. In the intravenous injections the solution reaches the heart directly, the coronary arteries thus receiving a large share and in the greatest concentration of solution; while in injections through the carotid artery the solution is first mixed with a great part of the blood of the entire body before it reaches the heart, and besides a good part of the solution while passing through the capillaries is given up to the tissues before it reaches the heart.

Calcium affects the heart as well as the vital centers in the medulla. Moreover, death from calcium may result from intravascular clotting of blood. It is probably due, in part at least, to this multiplicity of causes of death, that in the infusions with calcium there was a greater individual variability in the fatal doses than in the experiments with the other salts. However, in the calcium infusions, too, injections by the intra-arterial method seemed to be slightly more toxic than by the intravenous methods.

In the present series of experiments we estimated the toxicity of each tested salt by its fatal action on the entire animal. This fact has to be specially emphasized. Had we selected a single organ or a tissue as a test-object for our experiments, we might have found another order for the toxicity of the investigated salts.

Thus, for instance, if we had studied the effects of the very same salts upon the heart, the results would probably have shown that potassium was more toxic than magnesium. Or, if we had studied the effect of these salts upon muscle tissue, we might have found that calcium is more toxic than magnesium. Or, to cite another instance, if the eggs of the fundulus had served us as a test-object, we might have come to the conclusion that sodium is more toxic than potassium. The surviving, spontaneously beating heart of frogs or mammals is a most convenient organ for the study of the effects of substances upon living tissue. It served and serves therefore as a test-object in many of the pharmacological studies. Perhaps it is

due to this fact that the impression went abroad that potassium is the most toxic of the four chlorides under discussion. The central nervous system, however, is probably a more sensitive organ than the heart. Besides, the nerve tissues, or some of them, might have a special susceptibility towards the action of magnesium. By introducing, therefore, any of these salts into the circulation of the entire animal the organism reacts to it with that tissue or organ which is most susceptible to the particular substance. The respiratory mechanism apparently has a greater susceptibility towards magnesium than any other vital mechanism or organ has towards this or any other of the substances we have tested. Hence the greatest toxicity of magnesium in our experiments.

The order of toxicity which we have established for the four investigated salts, therefore, holds good only for their action upon the entire living animal. We may go even further in our restriction and state that it holds good only for the dog, the only animal which we have systematically investigated. For invertebrates or even for some vertebrates, the frog, for instance, the order of toxicity even for the entire animal might indeed be a different one.

A further analysis of our results brings to light a fact which seems to us to be quite instructive. We have seen that magnesium, calcium, and potassium are toxic in relatively small quantities. Now each of these substances is present in the living animal body in quite large quantities. The reason for their innocuousness is apparently that for the most part they are fixed within solid or semi-solid bodies, where they exert their specific activity: potassium essentially in muscles and red blood corpuscles and calcium and magnesium chiefly in the bones. The toxicity of these substances begins only when they reach the liquids of the body, especially the circulating medium, where they have a chance to come in contact with tissues and organs which are especially susceptible to their action. Now, the quantities of these inorganic substances within the blood or rather within the serum of dogs are, according to the determination of Abderhalden<sup>14</sup> as follows: Each kilogramme of serum contains  $\text{MgO}$ —0.04;  $\text{CaO}$ —0.113;  $\text{K}_2\text{O}$ —0.226;  $\text{Na}_2\text{O}$ —4.263; which means that each kilo-

<sup>14</sup> Abderhalden: *Zeitschr. f. physiol. Chemie*, vol. 25, p. 65.

gramme of serum contains Mg—0.024, Ca—0.0808, K—0.1876 and Na—3.164. This means, in other words, that magnesium is present in the smallest amount, calcium in the next larger amount and potassium in a still larger amount, while the quantity of sodium present in the serum is by many times larger than all of them. Now the interesting fact is that the order of the quantities in which these inorganic substances are present in the serum is exactly the same as that in which they proved to be toxic for the dog.

This parallelism comes out in a striking way when both data are reduced to comparative figures and are placed side by side. In the following table magnesium, existing in the smallest quantity in the serum and having the smallest fatal dose, is taken as 1, and the other three substances are given in the multiples in which they exist in the serum of dogs and as they proved to be fatal to these animals:

TABLE V.

SUBSTANCES.	EXISTING IN THE BLOOD IN RELATIVE CONCENTRATION.	FATAL IN RELATIVE DOSES.
Magnesium . . . . .	1.00	1.00
Calcium . . . . .	3.36	2.80
Potassium . . . . .	7.81	4.26
Sodium . . . . .	131.38	25.54

In comparing the two lines of proportions the following points ought to be borne in mind. Abderhalden's analyses were derived from two dogs only; an analysis of the sera of many animals might possibly have brought out figures still nearer those found by us for the toxicity. Furthermore, in infusion experiments the factor of elimination of the various salts has to be considered. Probably one salt is more rapidly eliminated through the kidneys and into the tissues than another, and the infused amounts of these salts do not give us an exact idea of how much of them is present in each case within the blood in a given unit of time, which alone is the precise measure of the fatal dose. For instance, if magnesium is eliminated from the blood more rapidly than calcium, an infused dose of calcium 2.8 times that of magnesium might mean in the end, as far as their relative presence in the serum is concerned, perhaps indeed a coefficient of 3.36. Again

biological experiments cannot be carried out with physical precision; for instance, it is not possible even with the greatest care to control perfectly the rapidity with which the solutions are injected intravascularly. In the infusion of sodium chloride the factor of the rapidity has certainly greatly influenced our results. We have already stated above that we had to inject the solution at a more rapid rate than the solutions of the other salts, in order to be able to bring an experiment to an end in one day. Had the injections been made more slowly, no doubt larger quantities could have been injected and the coefficient for the fatal dose of sodium might have been 50 instead of 25. Finally for sodium we ought to remember, as argued above, that with the large doses injected in molecular solution the physical factor of osmosis had surely a share in the fatal result, so that the chemical share alone in the toxicity of the sodium ion might indeed be 132 times smaller than the toxicity of the magnesium ion.

In brief, all these considerations should warn us against the expectation to find an exact similarity of figures in both kinds of comparisons. But there can be no doubt of the existence of a general parallelism between the proportions in which these salts are toxic to the entire animal (dog), and the proportion of their existence in the serum of that animal. Calcium is about three times less toxic than magnesium and is present in the serum in about three times the quantity of magnesium. Potassium exists in the serum in about double the quantity of calcium and is nearly one-half as toxic. Finally, sodium is present in the serum in great excess over the other three ions and is vastly less poisonous to the living animal. The law of toxicity of the four ions for the living entire animal can therefore be expressed by the statement **that the toxicity of magnesium, calcium, potassium and sodium to the entire animal is in inverse proportion to the amounts in which they are present in the serum of that animal. The smaller the amount of the ion in the serum, the more toxic it is in the infusion.**

This means, in other words, that *the less a vital tissue is exposed to the influence of an ion, the more it is susceptible to its toxic action.*

Whether the relations found here to exist in the dog will prove to hold true also for other animals, can be established only by direct studies of the underlying factors, *i. e.*, by a study of the comparative

toxicity of the inorganic constituents of the sera of these animals and by an analytical estimate of the normal concentration of these individual constituents within the serum. It is possible, for instance, that for the frog, for which animal, according to Binet, potassium is more toxic than magnesium, an analysis might reveal that in the serum of the frog, magnesium is indeed present in larger quantities than potassium. Correlations might also be discovered to exist between the relative abundance of magnesium salts in sea-water and the relatively low toxicity of the magnesium ion upon some marine animals.

As to the action of the substances which do not exist in the animal body, the toxicity of some of them might be found to stand in proportion to their chemical relationship to those substances which are constituents of the animal body.

#### SUMMARY

The physiological action of the chemical substance depends, in a great measure, on the living substance upon which it is to act. Effects found in actions on one tissue are not applicable, without special tests, to the other tissues of the same animal or to the same tissues of other animals. Effects found for simple tissues are not applicable to complex organs and effects found for organs are not applicable to entire animals.

The order of toxicity of the four chlorides under discussion was found in experiments on animals (dogs) to be magnesium, calcium, potassium, sodium. Magnesium was twice as toxic as calcium, potassium three times less toxic than the latter, while sodium was many times less toxic than any of the others. The dominant view that potassium is the most toxic was derived mostly from experiments upon the isolated heart.

For the dog, and perhaps for all mammals, the toxicity of the alkalis and alkali earths existing as constituents of the animal body is in inverse proportion to the quantities in which they are present in the *serum* of that animal; the smaller this quantity the more toxic it is in intravenous injections.

The convulsions produced by intravenous injections of sodium

chloride may be toxic in character and have the same origin as the twitchings and rhythmical contractions observed in frog muscles when immersed in solutions of sodium chloride. On the other hand, the early stoppage of the respiration might have its cause in the curare-like action which sodium chloride exercises upon the motor nerve endings.

## **THE EFFECT OF CERTAIN SO-CALLED MILK MODIFIERS ON THE GASTRIC DIGESTION OF INFANTS.**

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IN the fall of 1907, at the instigation of Dr. L. Emmett Holt, investigations were undertaken at the Rockefeller Institute in New York, in the hope of throwing light on some of the as yet obscure questions of infant digestion. The problem to be attacked was that of the digestion of milk in the infant's stomach, and more particularly the exact action of the more commonly used, so-called "milk modifiers," and their influence upon digestion both in health and disease.

A review of the literature of the gastric digestion of the infant<sup>1</sup> shows much valuable work on its physiology and its variations according to age and health. It has been shown that all the physiological factors occurring in the adult stomach are present in the infant, but in weaker form. It has further been proved that the younger the infant, the more active the motility of the stomach, that this motility is greater with woman's milk than with artificial food, and in health than in disease. It has been proved that the acidity of the gastric contents increases regularly after a meal for some time, but that free hydrochloric acid cannot be demonstrated for an hour or more after the ingestion of the food. This phenomenon has been shown to be due not to a lack of secretion of hydrochloric acid during the early stages of digestion, but to the striking power which casein has to combine with or adsorb it. Heubner has found that to 100 c.c. of cow's milk could be added 0.324 gram of hydrochloric acid before any test for the free acid could be obtained. Woman's milk will take up about one-half of that amount. It has further

<sup>1</sup> AMER. JOUR. MED. SCI., 1909, cxxxvii, 674.

been proved that it is that hydrochloric acid which is combined with the protein which acts in peptic digestion, and that only, while the occurrence of free hydrochloric acid is merely a sign that more of the acid is present than is required for complete digestion of the protein. Opinions differ as to the occurrence of lactic and volatile fatty acids in the child's stomach, while the presence of a fat-splitting enzyme has been both asserted and denied. Pepsin is always found in the infant stomach in both health and disease, and gastric digestion goes on to the formation of small amounts of peptones but no amino-acids. Rennin is always present after the first few weeks of life. Whether before that, or not, is a moot question.

For many years the medical profession has been adding barley water and lime water to cows' milk to increase its digestibility, and in more recent years sodium citrate and malt extract have been used extensively. There are many physicians who believe that each of these substances is at times of value in infant feeding, and each probably has his own rules by which he determines which is indicated in a particular case. Such knowledge, however, is largely empirical, as we have but little idea, or, at least, proof of what these substances do in the stomach and what the chemistry of their action really is. With the exception of some work of Einhorn twenty years ago, in which he investigated the motility and acidity of the gastric contents after feeding woman's milk, cow's milk and water, cow's milk and barley water, and several artificial foods, practically nothing has been done along these lines.

In these investigations, it was considered advisable first to use normal infants. This, however, not being practical, owing to the difficulty of obtaining such in a hospital, children who approached as nearly as possible a normal state of health were selected. All were patients at the Babies' Hospital in New York, and none were suffering from obvious digestion disturbances at the time of the examination. To obviate, as far as possible, the error due to individual variations it was attempted to take each case through a complete series of comparative examinations. In all 122 observations were made on 24 such infants, varying from two to eight months of age.

The test feedings given were of three series, designated Series I, II, and III. The purpose of Series I was to investigate the effect of the substances employed upon gastric secretion and for this purpose they were given with water alone. The three test feedings of this series consisted of a 5 per cent. solution of milk sugar—as an inert substance—barley water, and a 5 per cent. solution of lime water. In Series II, the substance was added to a mixture of cow's milk and water, equal parts. In this series was included woman's milk undiluted. It therefore embraced woman's milk, cow's milk and water, cow's milk and barley water, equal parts, cow's milk and water with 5 per cent. lime water, and cow's milk

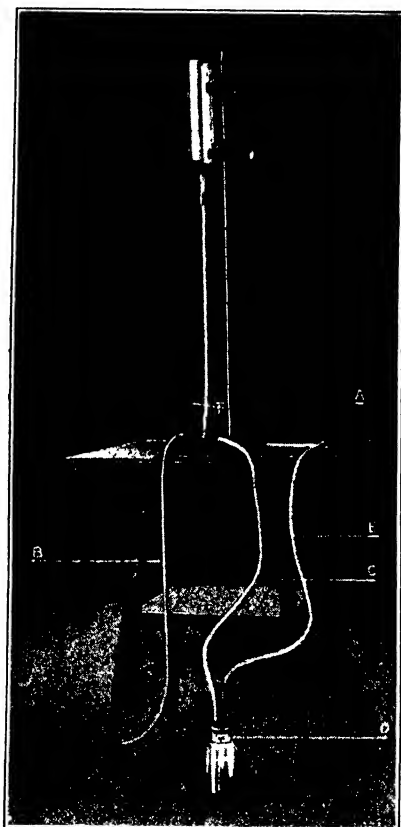
and water with 1 grain of sodium citrate to the ounce. In Series III, experiments were made with cow's milk in the dilution of one part in four, and lime water, barley water and sodium citrate were investigated. In nearly all the cases, the test feeding was given the first thing in the morning, six to eight hours after the preceding nourishment. In a few only was the stomach washed a half-hour before the feeding. Owing to the various times which the child took in ingesting the meal, it was deemed more reliable to take the time from the minute at which the feeding was done rather than from that of its beginning. This was done in every case.

On account of the difficulty sometimes encountered in removing the stomach contents of young infants by the ordinary method of stomach tube and funnel, and the necessity of working as rapidly as possible, a special apparatus was constructed for this purpose (see illustration). This consists of a graduated glass reservoir holding 500 c.c. of water, attached by a clamp to an iron upright so that it stands about two feet above the table. From the outlet, two to three feet of soft rubber tubing leads to a T-tube of large caliber. Just above the T-tube is a pinch cock (*a*). Over one arm of the T-tube is drawn the stomach tube, a soft rectal tube either 22 or 23 French (*b*). From the second arm of the T-tube runs a stiff tubing (*c*) to a bottle on the floor (*d*). The bottle fits into a wooden stand to keep it in an upright position and is supplied with a tightly fitting rubber stopper, through which protrude two glass tubes. The tubing (*c*) from the T-tube fits tightly over one, while from the second extends about four feet of similar stiff tubing (*e*). This last is held in the mouth of the operator and is used to apply gentle suction when for any reason the stomach contents do not siphon quickly. The bottle is detachable and is used as a specimen bottle.

In the investigation under consideration, the contents were removed without washing. The rubber stopper was then transferred to a second specimen bottle and, when desired, the stomach washed by alternately opening and closing the pinch cock at the same time with the fingers pinching and opening the tube (*c*) from the T-tube to the specimen bottles. This wash water was not used for investigation, and the lavage was performed merely to make it certain that most of the stomach contents had been obtained, or for therapeutic purposes. It was surprising to see how large a stomach tube a young infant could be made to swallow if held in an upright position in the nurse's arms, if no attempt was made to pass the pharynx until the child took a deep inspiration. The tube was passed with great ease and apparently with a minimum of discomfort. Most of the children, after having overcome the initial fright, and having become slightly used to the procedure, made very little effort to cry and some seemed quite content to suck on the tube. Though some of the babies failed to gain weight during the period of investigation

—possibly due to the frequent changes in the foods used—no serious or injurious results followed the procedure.

The specimens were taken at once to the chemical laboratories of the Rockefeller Institute and under the supervision of Dr. P. A. Levene, submitted to the following examinations: The quantity



Apparatus used to evacuate the stomach. (For description see the text.)

was carefully measured in order that by this means a rough estimate of the gastric motility might be obtained (A)<sup>2</sup>. The inaccuracy of drawing exact conclusions owing to the varying consistency of the milk curd and the uncertainty of complete evacuation is thoroughly appreciated. The acidity was estimated by titration with  $\frac{N}{10}$  NaOH, phenol-phthalein as an indicator (B). The hydrochloric acid in a

<sup>2</sup> Letters in parentheses refer to the columns in the tables in which the results are recorded.

free state was tested qualitatively with Congo red paper and quantitatively by titration against dimethylamidoazobenzol. The specimen used for the estimation of acidity, usually 5 c.c., after being neutralized was transferred to a platinum dish and evaporated over the steam bath to dryness. It was then carefully carbonized over a free flame, and the residue washed into a porcelain dish. In this, the chlorides were estimated by the method of Volhard. This result was calculated as parts per mille and recorded as "total chlorides" (C). A second portion of the contents was evaporated without the addition of NaOH, carbonized, and the chlorides estimated by the same method. This constituted the inorganic or fixed chlorides (D). The difference between the total chlorides and the sum of the fixed chlorides and free hydrochloric acid, shows the amount of chlorides which have gone into chemical combination with the milk proteins and are recorded as "organically bound chlorides" (E). It is this last item which is of importance in estimating the digestive capacity of various stomach contents when proteins are in the stomach. It has been shown by various authors that it is the combined chlorides only which act in peptic digestion, and that free hydrochloric acid in the stomach is merely a sign that more acid has been secreted than is required for complete digestion. The inorganic or fixed chlorides are, of course, inert. Acidity, fixed chlorides, and total chlorides will vary markedly with the food taken into the stomach, whereas the combined chlorides designate the exact amount which is being put to practical use. Tests were made in a number of cases for lactic acid, but as it was found in no case examined the proceeding was discontinued.

The method of estimating the digestive power of the gastric contents varied according to whether there were protein substances in the test meal or not. In Series I in which the "modifier" was given in water, without milk, the method was as follows: To each of the three portions of 5 c.c. of the contents was added 2 c.c. of fresh cow's milk. Portion A was untreated. To portion B was added  $\frac{n}{10}$  HCl until the casein had become saturated with the acid, as shown by the test for free acid with Congo red paper. Portion C was boiled before the milk was added, and then acidified in the same way as D. To each portion was added 1 c.c. of toluol. For A and B 25 c.c. measuring flasks were used; C requiring boiling, was placed in a small Erlenmeyer flask. All these portions were then placed in the incubator at body temperature for twenty-four hours. At the end of this time C was washed into a 25 c.c. measuring flask, by means of a saturated solution of zinc sulphate in five per cent.  $\text{H}_2\text{SO}_4$ . Enough solid zinc sulphate was added to each flask to saturate the solution, and the contents then raised in volume to 25 c.c. by adding the saturated zinc sulphate solution. The flasks were then placed in the ice box for twenty-four hours more.

This latter proceeding was to insure a constant temperature at which the coagulation took place regardless of the season. At the end of the twenty-four hours the specimens were filtered through folded filter papers. The amount of N in 10 c.c. of the filtrate was determined by the Kjeldahl process. The amount of nitrogen calculated for the 25 c.c. is designated as "peptone nitrogen." The amount of N in the filtrate of portion C (I) that portion which was boiled previous to the addition of the milk shows the amount of peptone and residual N in the given amount of contents and milk, and is used as a control, the amount being subtracted from the amount of N in the filtrates of A and B.

The amount of N, therefore, in the filtrate of A after the deduction of C gives the absolute amount of N in the form of peptones which were produced by the action of 5 c.c. of the stomach contents upon 2 c.c. of milk in twenty-four hours (J), whereas the N in the filtrate of B, similarly corrected by the deduction of C, gives the amount of peptone formation in twenty-four hours when the amount of acid is practically the same in all cases—in other words, an estimate of the amount, or at least actively, of the pepsin itself (K).

In Series II and III in which milk was given in the test meal the procedure was somewhat different. The contents were thoroughly mixed and then three equal parts measured out: when possible 15 c.c. in each part, when lack of material prevented, 10 c.c. One portion A was saturated at once with  $\text{ZnSO}_4$  and the volume raised to 25 c.c. by the saturated  $\text{ZnSO}_4$  solution. The second portion B was placed in the incubator with toluol for twenty-four hours and then saturated with  $\text{ZnSO}_4$ , while the third portion C was acidified with  $\frac{n}{10}$  HCl until the free acid showed a reaction to Congo paper, and then treated in the same manner as B. All three portions on being saturated with  $\text{ZnSO}_4$  were placed in the ice box for twenty-four hours and then treated in a similar manner to the specimen in Series I. The amount of N in the filtrate of portion A represents the peptones and residual N in the stomach at the end of an hour (G). This is, however, but a very rough estimate of the gastric digestion which has taken place, as it is open to variations due to the amount of peptone in the food and to the much greater variation dependent upon absorption and motility. The difference, however, between the amount of peptone at the end of the hour and that of the twenty-four hours more in the thermostat, while not designating the digestion which has taken place, in the stomach may be used as an indication of the digestive power of the gastric juice at the time of its removal and is designated as "increase in peptone nitrogen" (J). The difference between the peptone N of C and A by eliminating variations in acidity similarly estimates the activity of the pepsin in the stomach at the end of the hour (K).

Lastly to each of three small test tubes was added 1 c.c. of gastric contents, in Series I unfiltered, in Series II and III filtered. Tube

I was untreated (L); II was acidified with HCl until it showed bright red with dimethylamidoazobenzol (M); and III was boiled and acidified (N). The digestive power of these were then tested by the method of Mette, the digestion being estimated after twenty-four hours at body temperature.

These results were of use not only to confirm the previous findings but to show the presence or absence of "free pepsin," that is, pepsin not combined with the protein. The absence in every case of albumen digestion in the specimen which had been boiled and acidified (N) precluded the action of the acid in causing the digestion while the addition of toluol precluded bacteria.

Specimens of the test feedings as given to the patient were also examined for peptone N (Q), and total chlorides (P).

RESULTS. The results of these investigations are incorporated in tables of averages.

TABLE I.—*Normal infants. Investigation of lactose, lime water, and barley water administered in water, without milk. The specimen was removed from the stomach thirty minutes after the end of the feeding.*

	Number of cases	Amount of material removed from stomach		Total acidity	Total chlorides	Fixed chlorides	Free hydrochloric acid	Peptone nitrogen after 24 hours in the thermostat; untreated contents	Peptone nitrogen after 24 hours in the thermostat; acidified contents	Peptone nitrogen after 24 hours in the thermostat; boiled contents	Increase of peptone nitrogen; untreated contents	Increase of peptone nitrogen; acidified contents	Mette's tube tests; untreated contents	Mette's tube tests; acidified contents	Mette's tube tests; boiled contents
		c c.	° 30	° 30	° 30	° 30	mgr. mgr.	mgr. mgr.	mgr. mgr.	mgr. mgr.	mgr. mgr.	mgr. mgr.	mm. mm.	mm. mm.	mm. mm.
		A	B	C	D	E	G	H	I	J	K	L	M	N	
5% lactose solution	11	37	0 23	0 40	0 24	0 16	2 3	4 3	1 5	0 8	3 8	0 32	2 79	0	
Lime water, 5 parts water, 95 parts	15	36	0 31	0 61	0 41	0 20	3 1	4 6	1 2	1 9	3 4	1 07	3 46	0	
Barley water	12	50	0 34	0 82	0 69	0 13	2 6	5 4	1 3	1 3	4 1	0 35	3 00	0	

*Healthy Infants.* Examining first Series I (Table I), in which the substance to be investigated was given with water only, the following facts will be noted: Using the sugar and water as a control, it is seen (A) that the amount of material obtained was smallest after sugar and water and lime-water, and larger after barley water. The total acidity (B) is higher after both lime water and barley water than after a plain milk sugar solution; the total chlorides (C) are higher after lime water than after sugar and highest of all after barley water; while the fixed or inorganic chlorides (D) vary in about the same ratio, leaving, however, most free HCl (F)

after lime water and least after barley water. From these results, it would appear that any increase in chlorides following the administration of barley water is due to the extra salt given in the food, while when the lime water is used, an absolute increase of hydrochloric acid secretion results.

Examining now the digestive power of the gastric juice, it will be seen (J) that 5 c.c. of the stomach contents was able to produce in twenty-four hours from 2 c.c. of cow's milk very little peptone after sugar, more after barley water, and most after lime water, while the next column, that in which the acidity was equalized to demonstrate the relative amount of pepsin (K), the variations are much less in proportion, but show, if anything, the greatest amount of pepsin after barley water. The Mette's tube experiment, when unacidified (L), confirms the relative digestive powers of the different contents (J), while the next column (M), the Mette's tube acidified, tends to show again that the amount of pepsin varies but slightly.

In Series II (Table II), that in which woman's milk undiluted and equal parts of milk and water were given in the test feeding, the amount obtained (A) after feeding woman's milk is distinctly higher than after the other feedings, which here include sodium citrate, one grain to the ounce. The total acidity (B) is practically the same in all cases though somewhat higher after each of the three "modifiers" than after the plain milk. The total chlorides (C) are again higher after barley water and lime water, but slightly lower after sodium citrate than after the plain milk. A marked variation appears in the fixed chlorides (D). Taking the milk and water as a standard, the fixed chlorides, after a feeding including lime water, barley water, and sodium citrate are distinctly raised. The increase after barley water is again explainable by the larger amount in the food (P), the other three feedings showing practically the same salt contents. The low total chlorides and fixed chlorides after woman's milk are also explicable from the smaller amount found in the food. There being in no case free HCl (F) found in these rich milk diets, the difference between the total and fixed chlorides was the active organically bound chlorides (E). This shows that, though after lime water a certain amount of the hydrochloric acid was converted into calcium chloride and rendered inert, nevertheless a sufficient excess of the acid was secreted to raise that organically combined with the casein to the normal and to fulfill the demands of digestion. After barley water, though the excess of chlorides was due in a large part to that given in the food, a slight increase of combined chlorides is shown. After sodium citrate, the sodium bound the greater part of the HCl, while no stimulation to a further increase was offered, thus leaving a minimum of acid to act in digestion.

TABLE II.—Normal infants. Investigation of woman's milk and of lactose, barley water, and sodium citrate administered in equal parts of cow's milk and water. Time in stomach, sixty minutes after the end of the feeding.

	Number of cases		Amount of material removed from stomach		Total acidity		Total chlorides		Fixed chlorides		Organically bound chlorides		Free hydrochloric acid		"Peptone nitrogen" immediately		"Peptone nitrogen" after 24 hours in the thermostat, unfermented specimen		"Peptone nitrogen" after 24 hours in the thermostat, acidified specimen		Increase in peptone nitrogen; un-fermented specimen		Increase in peptone nitrogen, acidified specimen		Mette's tube tests; unfermented contents		Mette's tube tests; acidified contents		Chlorides in the food		"Peptone nitrogen" in the food																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
	c c	A	B	C	D	E	F	G	H	I	J	K	L	M	N	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	w	x	y	z	aa	ab	ac	ad	ae	af	ag	ah	ai	aj	ak	al	am	an	ao	ap	aq	ar	as	at	au	av	aw	ax	ay	az	ba	bb	bc	bd	be	bf	bg	bh	bi	bj	bk	bl	bm	bn	bo	bp	bq	br	bs	bt	bu	bv	bw	bx	by	bz	ca	cb	cc	cd	ce	cf	cg	ch	ci	cj	ck	cl	cm	cn	co	cp	cq	cr	cs	ct	cu	cv	cw	cx	cy	cz	da	db	dc	dd	de	df	dg	dh	di	dj	dk	dl	dm	dn	do	dp	dq	dr	ds	dt	du	dv	dw	dx	dy	dz	ea	eb	ec	ed	ee	ef	eg	eh	ei	ej	ek	el	em	en	eo	ep	eq	er	es	et	eu	ev	ew	ex	ey	ez	fa	fb	fc	fd	fe	ff	fg	fh	fi	fj	fk	fl	fm	fn	fo	fp	fq	fr	fs	ft	fu	fv	fw	fx	fy	fz	ga	gb	gc	gd	ge	gf	gg	gh	gi	gj	gk	gl	gm	gn	go	gp	gq	gr	gs	gt	gu	gv	gw	gx	gy	gz	ha	hb	hc	hd	he	hf	hg	hh	hi	hj	hk	hl	hm	hn	ho	hp	hq	hr	hs	ht	hu	hv	hw	hx	hy	hz	ia	ib	ic	id	ie	if	ig	ih	ii	ij	ik	il	im	in	io	ip	iq	ir	is	it	iu	iv	iw	ix	iy	iz	ja	jb	jc	jd	je	jf	jj	jk	jl	jm	jn	jo	jp	jq	jr	js	jt	ju	jv	jw	jx	ky	kz	la	lb	lc	ld	le	lf	lg	lh	li	lj	lk	ll	lm	ln	lo	lp	lq	lr	ls	lt	lu	lv	lw	lx	ly	lz	ma	mb	mc	md	me	mf	mg	mh	mi	mj	mk	ml	mn	mo	mp	mq	mr	ms	mt	mu	mv	mw	mx	my	mz	na	nb	nc	nd	ne	nf	ng	nh	ni	nj	nk	nl	nm	nn	no	np	nq	nr	ns	nt	nu	nv	nw	nx	ny	nz	oa	ob	oc	od	oe	of	og	oh	oi	oj	ok	ol	om	on	oo	op	oq	or	os	ot	ou	ov	ow	ox	oy	oz	pa	pb	pc	pd	pe	pf	pg	ph	pi	pj	pk	pl	pm	pn	po	pp	pq	pr	ps	pt	pu	pv	pw	px	py	pz	qa	qb	qc	qd	qe	qf	qg	qh	qi	qj	qk	ql	qm	qn	qo	qp	qq	qr	qs	qt	qu	qv	qw	qx	qy	qz	ra	rb	rc	rd	re	rf	rg	rh	ri	rj	rk	rl	rm	rn	ro	rp	rq	rr	rs	rt	ru	rv	rw	rx	ry	rz	sa	sb	sc	sd	se	sf	sg	sh	si	sj	sk	sl	sm	sn	so	sp	sq	sr	ss	st	su	sv	sw	sx	sy	sz	ta	tb	tc	td	te	tf	tg	th	ti	tj	tk	tl	tm	tn	to	tp	tq	tr	ts	tt	tu	tv	tw	tx	ty	tz	ua	ub	uc	ud	ue	uf	ug	uh	ui	uj	uk	ul	um	un	uo	up	uq	ur	us	ut	uu	uv	uw	ux	uy	uz	va	vb	vc	vd	ve	vf	vg	vh	vi	vj	vk	vl	vm	vn	vo	vp	vq	vr	vs	vt	vu	vv	vw	wx	wy	wz	xa	xb	xc	xd	xe	xf	xg	xh	xi	xj	xk	xl	xm	xn	xo	xp	xq	xr	xs	xt	xu	xv	xw	xx	xy	xz	ya	yb	yc	yd	ye	yf	yg	yh	yi	yj	yk	yl	ym	yn	yo	yp	yq	yr	ys	yt	yu	yv	yw	yx	yy	yz	za	zb	zc	zd	ze	zf	zg	zh	zi	zj	zk	zl	zm	zn	zo	zp	zq	zr	zs	zt	zu	zv	zw	zx	zy
Woman's milk, undiluted	5	100	97.0	88.0	37.0	51.0	00	53	81	104	28	51	0	1.08	0	0.20	41																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																											</			

A study of the amount of peptone N found in the stomach at the end of the hour (G) shows, with the exception of woman's milk, whose high content is due to the large amount of residual nitrogen in the food (Q), approximately similar results, with the lime water slightly the highest, and sodium citrate a shade low. The increase of peptone N during twenty-four hours in the incubator (J) shows that the digestive power following milk and water, lime water, barley water, and sodium citrate is about equal. The increase of peptone N after acidification with HCl and twenty-four hours in the thermostat (K) shows practically similar amounts of pepsin following cow's milk and water and cow's milk and barley water, whereas after sodium citrate and lime water it is a trace lower. This low figure after lime water is difficult to explain as it is at variance with all the other results. The amount of free pepsin, as shown by the Mette's tubes, is practically the same in all except woman's milk and barley water, which are low.

TABLE III.—*Normal cases Investigation of lactose, barley water, and sodium citrate, administered in cow's milk, 1 part, water, 3 parts. Time in the stomach, sixty minutes after the end of the feeding.*

		Number of cases	Amount of material removed from stomach	Total acidity	Total chlorides	Fixed chlorides	Organically bound chlorides	Free hydrochloric acid	Mette's tube tests; untreated contents	Mette's tube tests; acidified contents	Mette's tube tests; boiled contents
		C	A	$\frac{0.1}{100}$ B	$\frac{0.1}{100}$ C	$\frac{0.1}{100}$ D	$\frac{0.1}{100}$ E	$\frac{0.1}{100}$ F	mm. L	mm. M	mm. N
Cow's milk, 25% in water	7	36	0.75	1.33	0.64	0.65	0.04	0	2.66	0	
Cow's milk, 25% in water, 5% lime water	7	30	0.97	1.27	0.60	0.64	0.03	0	2.50	0	
Cow's milk, 25% in barley water	8	23	1.13	1.67	0.84	0.70	0.13	0	2.86	0	
Cow's milk, 25% in water, sodium citrate, 0.2%	5	21	0.71	1.09	0.92	0.18	0.00	0	2.55	0	

The third series of experiments as shown in Table III, that in which the substances under investigation were given with one part of milk in four, tend partially to confirm the findings of Series I and II. Lime water and barley water increase the total acidity (B), while the amount of free HCl (F) is too minute for consideration, though it should be noted that sodium citrate is the only substance used in this series, after which no free HCl was demonstrated in the gastric contents. The total chlorides (C) are highest after barley water and lowest after sodium citrate, while the fixed chlorides are highest after sodium citrate, leaving again an equality of combined chlorides (E) after milk and water and lime water and barley, with

a great decrease after sodium citrate. The figures demonstrating peptic digestion in this series are not very trustworthy, as the small amount of stomach contents usually obtained precluded thorough investigation of this point, and they are therefore omitted.

It would be inadvisable to attempt to draw definite conclusions from these series of experiments owing to the well known fallacies of averages in physiological work. A few facts, however, seem to be sufficiently suggestive to warrant their mention. It would appear, from the fact that in every case the addition of acid to the contents caused a greater increase of peptone formation than in the specimens in which the acid was not used, that when a deficiency of protein digestive power exists, it is due to a lack of acid rather than any want of pepsin. There is always more than enough pepsin in proportion to the amount of acid. As to the effects of the various substances investigated, it would appear that barley water has but slight, if any, effect upon HCl secretions. Lime water although usually given with the idea of reducing the acidity of the stomach may have quite the opposite result. Though a portion of the HCl found in the stomach is neutralized by the lime, the alkali stimulates to an increased secretion of HCl which may raise the acidity even higher than that obtained with milk alone. Sodium citrate, on the other hand, would appear to act in quite the opposite manner for, though preventing coarse curds, it converts most of the HCl into sodium chloride, liberating citric acid—a substance considered of little value in digestion—and does not stimulate further secretion of HCl. It would thus appear to weaken the protein digestive power of the gastric juice by using up its hydrochloric acid.

**PATHOLOGICAL CASES.** Having learned these facts concerning the physiology of the infant stomach and the effect of lime water, barley water, and sodium citrate upon the gastric secretion in health, it seemed advisable to undertake investigations upon cases suffering from digestive and nutritional disorders, in order to learn to what extent their gastric secretion varied from the normal and if possible to ascertain whether these various substances acted upon the diseased stomach in the same manner as upon the healthy organ. For this purpose two classes of cases were examined—marasmus cases without vomiting, and persistent chronic vomiting cases. Of the first class 14 cases were studied and 35 examinations were made, while of the chronic vomiting cases, 10 infants were used and 30 meals extracted. In these cases the test feeding given consisted of lactose, barley water, or lime water, given in water only, in the same concentration as was used with the healthy infants.

TABLE IV.—*Marasmus cases. Investigation of lactose, lime water, and barley water, administered in water without milk. Time in the stomach, thirty minutes after the end of the feeding.*

	Number of cases	Amount of material removed from stomach												
		c. c.	$\frac{0.1}{100}$	$\frac{0.1}{100}$	$\frac{0.1}{100}$	$\frac{0.1}{100}$	mgr.	mgr.	mgr.	mgr.	mgr.	mm.	mm.	mm.
		A	B	C	D	E	G	H	I	J	K	L	M	N
		Total acidity												
		Total chlorides												
		Fixed chlorides												
		Free hydrochloric acid												
		"Peptone nitrogen" after 24 hours in the thermostat; untreated contents												
		"Peptone nitrogen" after 24 hours in the thermostat; acidified contents												
		"Peptone nitrogen" after 24 hours in the thermostat; boiled contents												
		Increase of peptone nitrogen, untreated contents												
		Increase of peptone nitrogen; acidified contents												
		Mette's tube tests; untreated contents												
		Mette's tube tests; acidified contents												
		Mette's tube tests; boiled contents												
5% lactose solution	14	20	0.28	0.34	0.21	0.13	3.5	5.7	1.6	1.9	4.1	0.22	3.62	0
Lime water, 5 parts; water, 95 parts	9	25	0.33	0.50	0.32	0.21	3.2	4.4	1.9	1.6	2.5	1.16	3.22	0
Barley water	8	30	0.40	1.46	1.19	0.20	3.4	5.0	1.7	1.7	3.3	1.33	3.84	0

TABLE V. (Compiled from Tables I, IV, and VI).—Table showing the differences between the gastric contents of the various classes of infants studied. The test feedings consisting of a 5% solution of lactose in water. The stomach contents were removed thirty minutes after the end of the feeding.

	Number of cases	Amount of material removed from stomach												
		c. c.	$\frac{0.1}{100}$	$\frac{0.1}{100}$	$\frac{0.1}{100}$	$\frac{0.1}{100}$	mgr.	mgr.	mgr.	mgr.	mgr.	mm.	mm.	mm.
		A	B	C	D	E	G	H	I	J	K	L	M	N
		Total acidity												
		Total chlorides												
		Fixed chlorides												
		Free hydrochloric acid												
		"Peptone nitrogen" after 24 hours in the thermostat; untreated contents												
		"Peptone nitrogen" after 24 hours in the thermostat; acidified contents												
		"Peptone nitrogen" after 24 hours in the thermostat; boiled contents												
		Increase of peptone nitrogen, untreated contents												
		Increase of peptone nitrogen; acidified contents												
		Mette's tube tests; untreated contents												
		Mette's tube tests; acidified contents												
		Mette's tube tests; boiled contents												
Normal cases	11	37	0.23	0.40	0.24	0.16	2.3	4.3	1.5	0.8	3.8	0.32	2.79	0
Marasmus cases	14	20	0.28	0.34	0.21	0.13	3.5	5.7	1.6	1.9	4.1	0.22	3.62	0
Hypoacidity vomiting cases	4	40	0.15	0.19	0.09	0.08	2.3	3.9	1.7	0.6	2.2	0.08	3.50	0
Hyperacidity vomiting cases	6	28	0.44	0.52	0.20	0.33	4.5	6.9	2.1	2.4	4.8	1.61	4.55	0

The *marasmus* cases (Table IV) show very slight variation from the normal infants (Table V). The amount of material obtained

(the stomach was here evacuated thirty minutes after the child had finished its feeding) was slightly less than in the normal, whereas the total acidity and free HCl were practically equal in the two series. The increase chloride secretion following the administration of lime water shows quite as well in these cases as in the healthy children. It is of interest to note that the peptic activity in these cases is not reduced, appearing to be even a little greater than in the normal infants.

TABLE VI.—*Chronic vomiting cases. Investigation of the gastric contents in the two classes of this condition, and the effect of lactose, lime water, and barley water upon them. Time in stomach, thirty minutes after the end of the feeding*

	Number of cases	Amount of material removed from stomach		Total acidity	Total chlorides	Fixed chlorides	Free hydrochloric acid	"Peptone nitrogen" after 24 hours in the thermostat; untreated contents		"Peptone nitrogen" after 24 hours in the thermostat; acidified contents		"Peptone nitrogen" after 24 hours in the thermostat; boiled contents		Increase of peptone nitrogen; untreated contents	Increase of peptone nitrogen; acidified contents	Mette's tube tests; untreated contents	Mette's tube tests; acidified contents	Mette's tube tests; boiled contents
		c c. A	$\frac{0}{100}$ B	$\frac{0}{100}$ C	$\frac{0}{100}$ D	$\frac{0}{100}$ E		mgr. G	mgr. H	mgr. I	mgr. J	mgr. K	mm L	mm. M	mm. N			
<i>Hypoacidity cases</i>																		
5% lactose solution	4	40	0.15	0.19	0.09	0.08		2.3	3.9	1.7	0.6	2.2	0.08	3.50	0			
5% lime water solution	3	34	0.06	0.40	0.37	0.01		1.6	3.8	1.2	0.4	2.6	0.00	3.20	0			
Barley water	2	33	0.27	0.55	0.42	0.06		3.5	4.3	1.9	1.6	2.4	0.00	4.42	0			
<i>Hyperacidity cases</i>																		
5% lactose solution	6	28	0.44	0.52	0.20	0.33		4.5	6.9	2.1	2.4	4.8	1.61	4.55	0			
5% lime water solution	6	23	0.39	0.60	0.30	0.31		3.2	4.4	1.7	1.5	2.7	1.05	3.52	0			
Barley water	4	38	0.39	0.77	0.54	0.21		3.2	4.2	2.1	1.1	2.1	0.59	3.58	0			

In the *chronic vomiting cases* (Table VI), of which ten were studied, it was early seen that they naturally fell into two classes. In four of them the acid contents was distinctly subnormal and in the other six it was exceptionally high. In the *hypoacidity vomiting cases* the total acidity (B) was very slight and free HCl (F) either absent or present only in minute traces. These cases responded slightly to the administration of lime water, as is shown by the higher total chloride content of the stomach, but not sufficiently to neutralize the lime, and the free HCl was present in a trace only in one case. The peptone-forming power of the untreated stomach contents was somewhat low, while the action on the Mette's tubes was practically nil (L). That the failure of digestive power was due to a lack of acid,

and not to any deficiency of pepsin is shown by the strong action in the Mette's tubes after acidification of the specimens (M).

In the *hyperacidity vomiting cases* (Table VI) the total acidity (B) and free HCl (F) were distinctly high after all the test diets, the total chlorides and fixed chlorides varying in the same ratio as in the normal. The peptone formation from milk was also distinctly above normal (J) as was the Mette's tube estimations. The lack of an increase of digestive power of the acidified specimen, with the single exception of the milk sugar specimens, shows that this increase of proteolysis is again due to an increased acidity and not to any increase of pepsin.

Finally to learn whether these effects of the salts used in infant feeding occurred in all cases, a short series of experiments were made in which 18 unselected infants, the entire content of a ward, were examined for the acidity and chlorides (Table VII). In these cases the test feeding consisted of two parts of cow's milk and one part of water, and the substances used were lime water, 5 per cent., sodium citrate 0.2 per cent., and potassium carbonate 0.1 per cent., with sufficient lactose to make the carbohydrates 6 per cent. The meals were extracted one hour after the child received the bottle, in this respect differing from the other experiments. In this series the effect of lime water shown in the other experiments is not evident, but that of sodium citrate is fully confirmed by the increase of the fixed chlorides and consequent reduction in the organically combined chlorine. The potassium carbonate also increased the fixed chlorides and reduced the organic chlorine, but not so markedly as did the citrate. There was no increased stimulation of HCl by this salt.

TABLE VII.—*Unselected ill infants. Investigation of sodium citrate, lime water, and potassium carbonate administered in a mixture consisting of cow's milk, 1 part, water, 2 parts, and lactose sufficient to make 6%. (1.40-6-1.30.)*

	Number of cases	Amount of material re- moved from stomach	Total acidity	Total chlorides	Fixed chlorides	Organically bound chlorides	Free hydrochloric acid
		c.c.	$\frac{0}{100}$	$\frac{0}{100}$	$\frac{0}{100}$	$\frac{0}{100}$	$\frac{0}{100}$
	A	B	C	D	E	F	
Cow's milk and water . . . .	18	46	0.85	0.98	0.29	0.69	0
Cow's milk and water; 5% lime water solution	17	51	0.78	0.94	0.24	0.68	0
Cow's milk and water; sodium citrate 0.2%	13	44	0.85	0.91	0.65	0.26	0
Cow's milk and water; potas- sium carbonate 0.1%	15	52	0.64	0.97	0.44	0.52	0

**REVIEW OF RESULTS.** While fully appreciating the danger of drawing definite conclusions from the results of physiological investigations when these results are based on averages of the findings, a review of the above tables would seem to suggest at least certain facts. The motility of the stomach appears to be in inverse proportion to the concentration of the milk in the solution. The greater the dilution the more rapidly the organ empties itself. Lime water itself appears not to act, as is generally accepted in practical pediatrics, by reducing the acidity of the child's stomach. While unquestionably neutralizing a portion of the hydrochloric acid, the alkali stimulates in a further secretion of gastric juice. This raises the amount available in the form of free acid or organically bound chlorine at least to the amount normally occurring when the lime water is not added, and may cause a distinct over-stimulation of the gastric glands sufficient to produce a slight relative hyperchlorhydria. The effect of barley water in the stomach appears, at least from the purely chemical viewpoint, to be very slight, neither stimulating nor retarding gastric secretion. The action of sodium citrate, however, is quite definite. It acts similarly to the alkalies in converting a large portion of the hydrochloric acid into sodium chloride, while at the same time it does not stimulate to a further secretion of acid. In this way it reduces decidedly the amount of hydrochloric acid in the stomach. How potent a factor this may be in the well known action of this salt in preventing the formation of curd in the infant stomach is an open question. It has been shown by several observers that in the young infant the amount of rennin is very slight and it is possible that most of the clotting is due rather to the action of the acid in the stomach than to the ferment.<sup>3</sup>

<sup>3</sup> That sodium citrate does act in inhibiting acid clotting is shown by the following simple experiment: Into each of eight Erlenmeyer flasks was placed 5 c.c. of fresh cow's milk and varying amounts of  $n/10$  sodium citrate solution. After sufficient distilled water had been added to make the volume of fluid the same in all the flasks the solutions were titrated with  $n/10$  HCl until clotting occurred. From the accompanying table it will be seen that each increase in the sodium citrate in the milk required a proportional increase of the HCl to produce the clot.

Flask	Cow's milk c.c.	$n/10$ sodium citrate added to milk c.c.	Distilled water added c.c.	Amount of $n/10$ HCl required for clotting c.c.	Increase in the quan- tity of HCl required for each increase of 1 c.c. sodium citrate c.c.
1	5	0	7	2.0	—
2	5	1	6	2.3	0.3
3	5	2	5	2.6	0.3
4	5	3	4	2.85	0.25
5	5	4	3	3.2	0.3
6	5	5	2	3.45	0.25
7	5	6	1	4.00	0.55
8	5	7	0	4.30	0.3

An examination of the individual cases treated with milk mixtures containing sodium citrate showed that in those cases in which the acidity of the stomach was low practically all the hydrochloric acid was converted into sodium chloride, while in those of high acidity

Protein digestion in the stomach of the young infant would appear to be very slight and such as does occur to be in fairly direct proportion to the amount of acid present.

**PRACTICAL APPLICATION.** A practical application of these results is suggested in connection with the class of infants who vomit persistently from birth. According to these results these cases, while showing no especial clinical differences, may be divided into two fairly definite groups according to the acidity of their gastric contents. If the hyperacidity found in the majority of these cases is the chief cause of their vomiting the fact of the excess of acid and its degree should be known in order that the treatment may have a rational basis. The administration and removal of a test feeding in a young infant is a simple procedure, the greatest difficulty being the finding of the most appropriate substance to be used. A simple five per cent. solution of lactose in water has shown the most striking differences between the acid content of the stomach of individual infants and appears to be the most suitable feeding to test the gastric contents. It might, however, be well to follow this test after a day by a second examination in which a mixture of cow's milk, one part to water two parts is used. By this means the stomach both in comparative rest and during active digestion may be studied and the amount of acid actually secreted, owing to the stimulation of the milk, determined. It seems probable that the results of such findings will give some important indication for treatment. When the acidity is low, a weak alkali to stimulate acid secretion or possibly a few drops of dilute hydrochloric acid will be indicated, whereas if an oversecretion of hydrochloric acid is found, either a higher protein meal, thereby binding more of the acid, or sodium citrate to convert the hydrochloric acid into sodium chloride may be found effective. The accurate knowledge of the degree of acidity may be of especial value in cases of congenital pyloric stenosis or pylorospasm. In the one case we have had the opportunity to examine, the gastric acidity was exceptionally high. This agrees with the findings of Knoepfelmacher, Engel, and others. If, as seems probable, the pylorospasm is due to the hyperacidity, an accurate knowledge of its degree must be of no little value in treatment.

**SUMMARY.** 1. The motility of the infant stomach varies inversely to the concentration of the food. The more dilute the food the more frequently may the feedings be given.

2. Lime water does not reduce the acidity of the gastric contents, the neutralizing of a portion of the acid being overcome by an

the amount of the fixed chlorides was increased by a fairly definite amount, namely 0.043 per cent. A calculation of the amount of hydrochloric acid which should theoretically combine with 0.2 per cent. sodium citrate shows that one would expect 0.061 per cent. Allowing for the increased dilution from the gastric juice it would seem that the 0.043 would approach rather nearly the theoretical amount and that, therefore, the combination of HCl with sodium citrate in the stomach closely follows chemical laws.

increased stimulation of hydrochloric acid by the gastric glands. This may even increase the amount of acid available for digestion.

3. Sodium citrate acts on the acid in the stomach converting it into sodium chloride and thus markedly reduces the "available hydrochloric acid."

4. Barley water seems to have no constant effect upon the chemistry of gastric digestion in the infant.

5. The type of infants who vomit persistently may be divided into two classes, hypoacidity and hyperacidity.

6. Test feedings should be given to this type of infants to determine to which class they belong.

7. A five per cent. milk sugar solution seems to be the most satisfactory feeding to determine fine differences in the gastric contents. This may be followed by a mixture of milk one part, water two parts, to determine to what extent the gastric glands are capable of responding to stimuli. For the lactose solution thirty minutes is the most satisfactory time to allow the feeding to remain in the stomach; for the milk mixture sixty minutes.

8. On purely theoretical grounds it would appear that when the acidity is low either small doses of alkalies or of hydrochloric acid are indicated while in hyperacidity sodium citrate holds out the best hope of benefit.

9. Protein digestion in the infant's stomach is slight and is proportional to the amount of hydrochloric acid in the organ.

I desire to express my thanks to Dr. L. Emmett Holt for the inspiration and the many valuable clinical suggestions given during the course of this work; to Dr. P. A. Levene for his aid in directing and overseeing the chemical side of the problem; and to Dr. Hemenway, resident physician, her assistants, and the nurses of the Babies' Hospital for their coöperation and kind assistance.



## THE PRODUCTION OF EDEMA

AN EXPERIMENTAL STUDY OF THE RELATIVE ETIOLOGIC IMPORTANCE OF  
RENAL INJURY, VASCULAR INJURY AND PLETHORIC HYDREMIA \*

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The investigation here presented had for its object the demonstration of the relative importance of vascular injury, renal injury and hydremia in the production of edema, and was suggested by a recent theoretical consideration<sup>1</sup> of the part played by chemical correlation in the pathologic conditions associated with disturbances of renal function.

Experimental evidence indicating the importance of hydremia or of vascular injury is not lacking, but, as it is based mainly on transfusion experiments, in which large amounts of fluid were used, and frequently experiments on dead or nephrectomized animals, it is not entirely satisfactory in that the conditions are too artificial. The recent observations on the edema of uranium nephritis point the way to a method of study which affords conditions more nearly in keeping with those associated with edema in man.

Uranium nephritis, in rabbits at least, is accompanied, when an excess of water is administered by the stomach-tube, as first shown by Richter,<sup>2</sup> by a well-marked edema of the subcutaneous tissues and by hydrops of the pleural and peritoneal cavities—a condition which several investigators have found not to occur when an excess of water is administered to animals poisoned with chromic salts, aloin and other renal irritants. This difference in effect would indicate that uranium nitrate has an action not common to the other renal poisons, and from our knowledge of edema it seems probable that this action is one injurious to the blood vessels. Of greater interest is the observation that the serum of an animal with uranium nephritis, when introduced into an animal with a chromate nephritis, causes the development of a well-

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1. Pearce (R. M.): The theory of chemical correlation as applied to the pathology of the kidney. *THE ARCHIVES INT. MED.*, 1908, ii, 77.

2. Richter (P. F.): Die experimentelle Erzeugung von Hydrops bei Nephritis, *Beitr. z. klin. Med. (Festschrift f. Senator)*, Hirschwald, Berlin, 1904; *Experimentelles über die Nierenwassersucht*, *Berl. klin. Wchnschr.*, 1905, xlii, 384.

marked edema. This phenomenon, first observed by Heineke<sup>3</sup> and since confirmed by Blanck,<sup>4</sup> who, however, finds it to be not a constant occurrence, suggests that in the serum of animals with nephritis there may occur substances which operate to produce edema. Two explanations seem possible: either the retention, as the result of kidney insufficiency, of substances which act as lymphagogues of the second order; or the injurious action on the endothelium of some substance or substances causing an alteration in its permeability to fluids. These several observations, therefore, suggest a new method of experimentation for determining the relative importance of hydremia, renal injury and vascular injury in the production of edema.

It is unnecessary, on account of the recent admirable presentation of the subject by Meltzer,<sup>5</sup> to go in detail into the question of the mechanistic *versus* the vitalistic theories of lymph formation. It is sufficient to recall that of the latter theories Heidenhain's, as well as Hamburger's, assumes an increased activity of the endothelial cells caused by katabolic products, and that Lazarus-Barlow and Asher believe in the influence of cell action, but of the cells of the organ rather than of the endothelia. Lazarus-Barlow further emphasizes the influence of waste products. Starling, who supports the purely physical theory, assumes an altered permeability of the endothelial membrane.

In connection with these theories of the physiology of lymph secretion, we have certain views concerning the pathologic secretion of lymph which point to vascular injury as an important factor. Cohnheim and Lichtheim,<sup>6</sup> in their well-known experiments on the production of hydremic plethora, found that the injection of large quantities of salt solution into the veins of rabbits and dogs, although it led to ascites and edema of the internal organs, did not cause edema of the normal skin and subcutaneous tissues; but if the skin was irritated, as by exposure to the sun, painting with iodine or immersion in hot water, local edema of the skin always followed transfusion. From these experiments Cohnheim concludes that the mild irritation of the skin caused an alteration of the capillary walls which made them more permeable for the fluid of the hydremic plethora. Support of this theory is offered by the experi-

3. Heineke, quoted by Müller (F.): Verhandl. d. deutsch. path. Gesellsch., 1905, ix, 64.

4. Blanck (S.): Experimentelle Beiträge zur Pathogenese des Nierenwassersucht, Ztschr. f. klin. Med., 1906, lx, 572.

5. Meltzer (S. J.): Edema. Am. Med., 1904, iii, 19, 59, 151, 191.

6. Cohnheim (J.) and Lichtheim (L.): Ueber Hydrämie und hydrämisches Oedem. Virchow's Arch. f. path. Anat., 1877, lxi, 106.

ments of Magnus,<sup>7</sup> who finds that edema of the skin occurs in transfused animals if previously arsenic, which pharmacologists consider a specific poison for blood vessels, is injected, or if animals are in deep anesthesia from chloroform or ether. Magnus also finds that in nephrectomized animals transfusion leads to anasarca. Somewhat similar results have been obtained by Albu.<sup>8</sup> Closely related to Cohnheim's theory of renal edema is that of Senator.<sup>9</sup> The difference is that Cohnheim assumes that the altered permeability of the capillary walls is due to the action on these structures of toxic substances not eliminated as the result of renal insufficiency. Senator assumes that the edema is as much primary as is the renal lesion, and that the two are caused by the same toxic agent affecting the glomeruli of the kidney as well as the vessels of the skin, the toxic agent having its origin in the primary disease, as scarlet fever, malaria or other conditions.

The edema-producing power of the serum of nephritis has been only recently studied, and the observations are few in number. Heineke's experience with the serum of uranium animals has been mentioned. This serum, from animals with edema, injected into animals poisoned with chromic salts, which, in Heineke's experience, does not cause edema, produced hydrops of the pleural and peritoneal cavities. His experiments were not reported in detail, but have been confirmed by Blanck, who, however, found that the condition could not be reproduced constantly.

In a later study with Meyerstein,<sup>10</sup> Heineke reports the production of edema in 64 per cent. of the animals receiving uranium serum intravenously; but he also found edema in 60 per cent. of those receiving normal rabbit serum. The animals of both groups had been poisoned for four to five days with sodium bichromate and had received water and sodium chlorid by the stomach-tube. As this treatment, in the absence of serum injection, did not cause edema, it is suggested that the serum in both instances had some injurious effect on the blood vessels. Of similar import are the results obtained by Georgopoulos,<sup>11</sup> who pro-

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7. Magnus (R.): Ueber die Entstehung der Hautödeme bei experimentelles hydrämischer Plethora. Arch. f. exper. Path. u. Pharmak., 1899, xlii, 250.

8. Albu: Zur experimentellen Erzeugung von Oedemen und Hydropsien. Virchow's Arch. f. path. Anat., 1901, clxvi, 87.

9. Senator (H.): Ueber die Wassersucht bei Nierenkrankheiten. Berl. klin. Wehnschr., 1895, xxxifi, 165.

10. Heineke (A.) and Meyerstein (W.): Experimentelle Untersuchungen tieber den Hydrops bei Nierenkrankheiten. Deutsch. Arch. f. klin. Med., 1907, xc, 101.

11. Georgopoulos: Experimentelle Beiträge zur Frage der Nierenwassersucht, Ztschr. f. klin. Med., 1906, lx, 411.

duced a moderate edema by injecting nephrectomized rabbits with the serum of animals suffering with uranium nephritis.

In this connection should also be mentioned the observations of Kast<sup>12</sup> and Starling<sup>13</sup> on the lymphagogenic action of the serum of edematous nephritics when injected into animals. Kast injected into the vein of a dog 75 cubic centimeters of the serum of a very edematous individual suffering from chronic hemorrhagic nephritis and found the flow of lymph to be increased tenfold. Sera from two other nephritics with edema increased the flow threefold and twofold, respectively, while the serum of normal individuals and of nephritics without edema gave no results, as was also the case in one instance, each, of uremia and cardiac dropsy. Starling reports a single experiment on the dog in which it was observed that the serum of a uremic individual caused a marked quickening of the flow of lymph from the thoracic duct.

My own experiments have had for their object the production, in the presence of artificial plethoric hydremia,<sup>14</sup> of edema by the administration of substances which would not only produce a renal lesion, but also injure the vessels of the body generally; but more especially the object has been to demonstrate ultimately the presence of endotheliotoxic substances in the serum of animals with experimental nephritis.

Experiments covering only the first part of this proposition are here presented. Rabbits have been treated with substances known to be both renal and vascular poisons, and hydremia has been produced by introducing through a stomach-tube a considerable amount of water. In other experiments a specific renal poison has been administered first, and later a vascular poison, or *vice versa*. The substances used have been potassium chromate, uranium nitrate, arsenious acid, ricin and snake venom. To these are added some very suggestive experiments with nephrotoxic immune serum which appear to indicate that this serum has definite endotheliotoxic properties.

The influence of salt retention as a factor has not been lost sight of, but no attempt has been made to determine the effect of the increased

12. Kast (A.): Ueber lymphagoge Stoffe im Blutserum Nierenkranken. Deutsch. Arch. f. klin. Med., 1902, lxxiii, 502.

13. Starling (E. H.): Physiologic factors involved in the causation of dropsy. Lancet, London, 1896, cl, 1407.

14. This term, "plethoric hydremia," is used to indicate an increase in the amount of water in the circulating fluid, without necessarily a decrease of the proteid constituents. The term "hydremia" is hardly appropriate, as it was originally used to indicate a dilute condition of the blood resulting from the withdrawal of protein by albuminuria. Wherever, therefore, the term "hydremia" is used in this paper it refers to condition of plethora due to increased ingestion of fluid.

administration of this substance. As primary salt retention has not been proved, it has been assumed that salt retention accompanies water retention and that, therefore, this factor would be constant in all experiments.

That this plan of study represents a somewhat crude way of approaching the problem is at once acknowledged. It seemed to me, however, that, by adopting a careful system of controls, information of value might be possibly obtained.

#### I. EXPERIMENTS WITH URANIUM NITRATE

The first experiments had for their object the confirmation of the observations of Richter and others, that edema is associated, when an excess of water is administered, with the nephritis caused by uranium nitrate. Such confirmation was readily obtained, as is shown in the following experiment.

*Rabbit 13*, weight 1,830 grams, on Jan. 30, 1908, and February 1 received 0.0075 grams uranium nitrate subcutaneously; on January 31 and February 1 and 2, 100 cubic centimeters of water were administered by stomach-tube. Albumin appeared in the urine on January 31 and increased in amount rapidly until February 3, when no urine was voided. On that date the animal was killed.

The examination showed well-marked edema of the subcutaneous tissues, about 15 cubic centimeters of clear fluid in the abdominal cavity and smaller amounts in each pleural cavity. The subpleural, subperitoneal and perirenal tissues were distinctly edematous. Especially noteworthy was the edema of the kidneys and their pelvis and ureters. The latter were greatly swollen and on cross-section presented a uniform glistening, gelatinous appearance, with apparently complete obliteration of their lumina. The bladder contained only 3 cubic centimeters of urine; microscopically this urine showed an abundance of fine granular casts, a few hyaline casts and leucocytes and epithelial cells.

In definite contrast to this is the absence of edema in animals receiving uranium, but no water; for example, the following:

*Rabbit 36*, weighing 2,410 grams, received on March 23, 24 and 25, 1908, 1 cubic centimeter of uranium subcutaneously. Albumin was present in the urine on March 24 and 25 and anuria developed on the 26th; the animal died on the 27th. No increase of fluid in either tissues or cavities of the body was found on postmortem examination.

Although similar negative results were obtained in four other animals thus treated, it must be admitted that Georgopoulos found varying grades of edema in three animals receiving uranium nitrate, but no water.

These experiments bring out the well-known relation of kidney injury and hydremia in the production of edema, but the relation of vascular injury is not so clear, though it is possible the latter was present and due to uranium nitrate. That this salt has an injurious effect on vascular structures is generally assumed, though it must be admitted

that the evidence on this point is not conclusive, being a matter of opinion rather than of actual observation.<sup>15</sup> Leaving this question for the present, we may turn to the experiments in which definite vascular poisons were used.

## II. EXPERIMENTS WITH ARSENIC

Arsenic, which pharmacologists consider a vascular as well as a renal poison, was employed in the early experiments. Nine rabbits were used; all received daily 0.5 to 1 cubic centimeter of a 1 per cent. solution of arsenious acid; six of these received also 50 to 100 cubic centimeters of water by the stomach-tube daily, and into the subcutaneous tissues of three of the latter potassium chromate was injected previous to the arsenic.

The animals receiving arsenic alone developed a localized edema at points of injection, but nowhere else. The following protocol illustrates this experiment:

*Rabbit 44*, weighing 1,700 grams, received, on March 25, 26, 27 and 28, 1908, 1 cubic centimeter of a 1 per cent. solution of arsenious acid subcutaneously. The urine contained only faint traces of albumin. No urine was voided on the 28th and 29th, and on the latter date the animal was killed. About the areas of injection and closely limited to these points the subcutaneous tissues were infiltrated with a slightly blood-tinged fluid which gave a gelatinous appearance. The peritoneal and pleural cavity contained no fluid. The bladder contained 5 cubic centimeters of thick urine which responded to tests for albumin.

The three rabbits receiving water by the stomach, in addition to arsenic subcutaneously, presented diffuse edema of varying grades, the most severe of which may be described as follows:

*Rabbit 43*, weighing 2,080 grams, received 1 cubic centimeter of arsenic solution subcutaneously and 100 cubic centimeters of water by stomach on March 25, 26, 27 and 28, 1908. Albumin appeared in the urine on the 27th; the 29th, when the animal died, no urine was voided. The subcutaneous tissues of the entire abdomen presented a diffuse edematous condition, with here and there petechial hemorrhages. The edema was not limited to points of injection but involved subcutaneous tissues of nether portion of body and extended into all four legs. Ten cubic centimeters of clear fluid were present in the peritoneal cavity and three in each pleural cavity. The bladder contained no urine.

The other two animals, in which anuria did not develop, presented a frank, but less extensive edema.

In the third group of three animals a chromate nephritis was first produced and then arsenic and water administered. The resulting edema was of about the same extent as in those receiving arsenic and water.

15. The recent observations of Schlayer, Hedinger and Takayasu (*Ueber nephritisches Oedem*, Deutsch. Arch. f. klin. Med., 1907, xci, 59) indicate that the lesions of uranium nephritis include an increased permeability of the cutaneous vessels following a decreased permeability of the glomerular vessels.

The first of these three groups of experiments illustrates a local edema, due to direct vascular injury, and the second and third the influence of hydremia, and presumably the greater diffusion of the vascular poison, in causing a general edema. In the third group, although two renal poisons were administered, the edema did not differ from that of the second group. This would indicate that possibly arsenic does not add to the lesion caused by the chrome salt, or that the degree of kidney injury is relatively unimportant.

### III. EXPERIMENTS WITH RICIN AND VENOM

Of special importance are the experiments in which ricin or venom was administered to rabbits with chromate nephritis and artificial hydremia. Neither ricin or venom has the power, except in large or rapidly repeated doses, of producing serious injury to the kidney. On the other hand, ricin has been found by several investigators to cause very severe vascular injury. This is brought out very prominently in Flexner's<sup>16</sup> study of the lesions caused by this substance. Venom (*crotalus adamanteus*) also seriously injures blood vessels, and, as shown by Flexner and Noguchi,<sup>17</sup> contains an endotheliolytic substance which these investigators termed "hemorrhagin." It was the influence of this endotheliolytic substance that was especially sought in the experiments with venom. Both substances, but especially venom, have a variety of toxic effects, but any possible influence which these may have on the production of edema I have attempted to control.

The rattlesnake venom used was obtained from Dr. Hideyo Noguchi, of the Rockefeller Institute, and the ricin from Prof. C. H. Bunting, of the University of Wisconsin, to both of whom I am greatly indebted, in that I was thus able without preliminary testing to use preparations the toxicity of which they had determined.

The chief experiments were very completely controlled by other experiments in which animals received (1) ricin alone, (2) water alone, (3) ricin and water, (4) potassium chromate alone, (5) potassium chromate and water, (6) potassium chromate and ricin. A similar set of controls was made with venom.

In each of four sets of experiments with ricin and four with venom this scheme was followed. In this way the results in the chief experiments were satisfactorily controlled by the other combinations. The

16. Flexner (S.): The pathology of toxalbumin intoxication. Johns Hopkins Hops. Rep., 1897, vi, 346.

17. Flexner (S.) and Noguchi (H.): The constitution of snake venom and snake sera. Univ. Penn. Med. Bull., 1902, xv, 345.

animals of the major experiments, receiving ricin (or venom), chrome and water, usually died on the fifth or sixth day; the others, for the sake of comparison, were killed at the same time. The ricin and venom were injected into the ear vein, the former in doses of 0.02 mg., the latter of 0.5 mg. of the dried venom on the first, second and fifth days. The potassium chromate was given subcutaneously in doses of 0.03 gram on the first and second days. Water, usually 100 cubic centimeters, was administered daily by stomach-tube. In some experiments this regularity in the administration of the chromate solution or the venom and ricin was varied.

Three of the four animals receiving ricin, potassium chromate and water developed a severe and widespread edema, as is seen in the following protocol. The fourth, dying on the third day, had an edema of less degree, with only a very small amount of fluid in the serous cavities.

*Rabbit 104*, weighing 1,695 grams, was isolated on Jan. 10, 1909. The urine of the 11th was normal and the animal received 0.02 mg. ricin in the ear vein, 0.03 gram potassium chromate subcutaneously, and 100 cubic centimeters of water by stomach-tube. This treatment was repeated on January 12 and again on the 15th. On the 13th, 14th and 16th water only was given. The urine of the 12th contained a trace of albumin and that of the succeeding days showed a severe albuminuria. Casts were abundant. Anuria did not develop and the animal was killed on the 17th. At the autopsy was found a well-marked edema of the subcutaneous tissues of the abdomen and thorax, 24 c.c. of fluid in the abdominal cavity and 16 c.c. in both pleural cavities. The retroperitoneal, perirenal and pelvic fat and tissue about the pancreas presented an extreme edema of characteristic gelatinous appearance; a similar condition existed in the retrosternal and mediastinal tissues.

In a second animal of this group, the subcutaneous tissues of the abdomen were swollen to a thickness of 0.8 cm. and the serous fluid dripped freely from the incision. The edema of the skin of this animal was so extreme that the hair could be readily scraped from considerable areas.

Similar results were obtained with animals receiving venom. In one animal, dying on the third day, the edema was limited to the subcutaneous tissue, but in the other three, living five or six days, it was of the general type shown in the following experiment:

*Rabbit 103*, weighing 2,230 grams, was isolated on Jan. 10, 1909. On the following day the urine was found to be normal and the animal received 0.5 mg. venom in the ear vein, 0.03 gram potassium chromate subcutaneously, and 100 cubic centimeters of water by stomach-tube. This treatment was repeated on January 12 and 15, with the administration of water only on the 13th and 14th. Albumin in small amount was found in the urine of the 12th and in large amounts on succeeding days; casts were abundant; no anuria. Death occurred on the morning of the 16th. Postmortem examination showed a moderate but very diffuse hemorrhagic edema of the subcutaneous tissues of abdomen and thorax, great distention of abdomen by ascites and 5 cubic centimeters of fluid in

each pleural cavity. The retroserous tissues generally, but especially retroperitoneal and mediastinal, were greatly distended by fluid. The pancreas lay in a gelatinous mass of edematous fat tissue. The ureters and pelvis of kidney showed a hemorrhagic edema which increased the cross-section of the upper ureter to a diameter of half a centimeter. A similar edema involved the perirenal fat. The kidney itself was edematous and had a peculiar mottled appearance due to extreme injection of the glomeruli. A few small hemorrhages were seen in the liver.

The control experiments follow:

1. *Ricin*.—This, given alone, causes no edema, though in two animals the peritoneal or pleural surfaces were unusually moist and barely 2 cubic centimeters of fluid could be drained from these cavities. No serious disturbance of the kidney occurs, though the urine occasionally contains a trace of albumin.

2. *Venom*.—After the administration of venom only, although ecchymotic hemorrhages are frequent, no edema has been found, except in one instance, in which a hemorrhagic edema of the ureters and pelves of kidneys occurred, with increased moisture of the serous membranes. The urine of this animal contained abundant albumin, numerous red blood corpuscles, and a few casts. In none of the other animals did the urine contain more than an occasional trace of albumin. Histologically, however, some of these kidneys showed exudative glomerular lesions, due apparently to the vascular injury caused by the venom.

3. *Water*.—The administration of water by the stomach-tube in daily amounts of 50, 75 or 100 cubic centimeters, according to the size of the animal, has not led to interstitial edema, though in two instances less than 3 cubic centimeters of clear fluid have been drained from the serous cavities of thorax and abdomen.

4. *Ricin and Water*.—In this series, in one animal 6 cubic centimeters of fluid were found in the abdominal cavity and 1 cubic centimeter in each of the pleural cavities. The other animals presented no evidence of edema. The moderate escape of fluid seen in one of these animals may represent a slight grade of the very extensive ascites which Flexner describes as the result of injecting intravenously large doses (0.1 to 3 mg.) of ricin and which also caused serious lesions of the kidney.

5. *Venom and Water*.—The results here, with the exception of one experiment, did not differ from those with venom only. The exception presents, however, one of the most marked examples of edema which has been met in the entire investigation and will be presented in detail.

*Rabbit 102*, weighing 1,590 grams, was isolated on Jan. 10, 1909. On January 11 its urine was found free of albumin and it received in the ear vein 0.5 mg. of venom and by stomach-tube, 100 cubic centimeters of water, which treatment was repeated on the 12th. On January 13 and 14 water only was given. On January

12 the urine was free of albumin, but on the 13th it was reddish in color and by the spectroscope was found to contain hemoglobin; albumin was present in considerable amount and the sediment contained numerous casts, red blood corpuscles and renal cells. No urine was passed on the 14th, and on the 15th, shortly after death, only a small amount, highly concentrated and containing a large amount of albumin; hematuria was still present. On postmortem examination was found a slight but readily demonstrable edema involving diffusely the subcutaneous tissues of the abdomen. The peritoneal cavity contained 24 cubic centimeters of bloody fluid and the pleural cavities about 10 each. The pelvis of both kidneys and first portion of both ureters were intensely edematous and hemorrhagic. The bladder contained 13 cubic centimeters of thick blood-tinged urine. Fat necroses were present in the abdominal fat generally and to less extent in that of pleura and pericardium with a few foci in the subcutaneous fat, especially that about the mammary glands. Microscopic examination of the kidney revealed a severe acute exudative glomerular nephritis.

Here was produced unexpectedly, as the result of a severe exudative nephritis due to the action of venom on the glomerular capillaries, the full picture of edema observed in animals receiving venom and water during a chromate nephritis. The essential element of kidney injury had been brought about in a peculiarly susceptible animal, and the control experiment became a major experiment, illustrating completely the thesis under consideration.

6. *Potassium Chromate*.—The subcutaneous injection of potassium chromate, in dose of 0.03 gm. daily for three days, produces a severe nephritis which the animal may survive, but which usually causes death in four to five days. Such animals present a definite gelatinous edema of the subcutaneous tissues immediately about the point of injection without tendency to become diffuse. No excess of fluid is found in the serous cavities. With animals receiving a single dose or two doses, and living a longer time, the result is the same.

6. *Potassium Chromate and Water*.—In this group the edema about point of injection was very definite and tended to involve the adjacent tissues to some extent. It did not, however, become diffuse, and in three of the animals no accumulations of fluid were found elsewhere. In a fourth, however, the abdominal cavity contained 4 and the pleural cavities 10 cubic centimeters of clear fluid and the retrosternal tissue showed a very definite edema. In the fifth animal of this series occurred an edema of such extent that it is deemed advisable to present the notes in full.

*Rabbit 100*, weighing 1,920 grams, was isolated on Jan. 10, 1909. The urine of the 11th was normal, and on this day the animal received 0.03 gram of potassium chromate subcutaneously and 100 c.c. of water by the stomach tube. The treatment was repeated on the 12th, and on the 13th, 14th and 15th water only was administered. Albumin appeared in the urine on the 12th and was present in large amounts on the 13th and 14th. No urine was voided on the 15th and until noon of the 16th, when the animal was killed by chloroform, the anuria

was complete. At autopsy was found a diffuse gelatinous edema of the subcutaneous tissues of the abdomen and thorax, which in some places measured a centimeter in thickness. Free fluid in considerable amounts could readily be squeezed from these tissues. The abdomen contained 11 cubic centimeters of clear fluid and the perirenal, retroperitoneal and pelvic fat, and that about the stomach and pancreas was distended as the result of an infiltration by a clear fluid, giving the tissues a jelly-like appearance. The pelves of the kidneys and the upper portion of the ureters were slightly edematous. Each pleural cavity contained a small amount of fluid and the retrosternal tissues were plainly edematous. The bladder contained only 4 cubic centimeters of urine.

Here, again, an experiment, planned as a control, differs from its fellows and becomes a positive experiment. The only possible explanation of vascular injury lies in the occurrence of anuria, which presumably allows the accumulation of non-eliminated substances capable of injuring vascular endothelium and causing edema.

8. *Potassium Chromate and Ricin*.—In these controls occurred a very intense edema at the point of injection of the chrome solution, and for a distance of several centimeters about this point, but not involving the abdomen generally and with no accumulation of fluid elsewhere in the body. The infiltration was, however, more extensive than that following chrome injections only, the result presumably of the added action of the ricin, on the blood vessels.

9. *Potassium Chromate and Venom*.—This combination caused a widespread hemorrhagic edema of the subcutaneous tissues of the abdomen encroaching in one animal on the thorax and in another extending to the tissues of the neck and proximal portions of extremities. In none, however, despite the presence of small hemorrhages of serous membranes, did fluid accumulate elsewhere.

These control experiments have been presented somewhat at length for two purposes: first, as fairly satisfactory controls of the preceding experiments, and, second, to illustrate some of the difficulties of controlling perfectly the three factors of plethoric hydremia, renal injury and vascular injury. It is evident that in most instances, with the doses here given, no one of these factors, or combination of two, is sufficient to produce a general condition which might be termed edema. Occasionally, however, it is difficult to interpret a small accumulation of fluid in one of the body cavities, and the value of the control is thus lessened. In this connection it is worthy of note that recently in laparotomies on a series of five rabbits with albumin-free urine I have twice observed the presence of a small amount of free fluid in the abdominal cavity. It is evident, therefore, that normal rabbits, under ether anesthesia at least, may occasionally present this condition. As has been seen, the third factor may at any time develop, as in Rabbit 102, in

which a severe nephritis unexpectedly complicated the experiment and led to a widespread condition of edema. Again, in Rabbit 100, an anuria, by allowing, presumably, the retention of products capable of vascular injury, was probably responsible for the unexpected edema.

It may also be pointed out that the various controls with chrome salts illustrate the circumscribed influence of a simple local injury. Chrome alone causes a purely local edema. When water is administered simultaneously, the edema is not so sharply circumscribed, but may still be considered as localized. When chrome and venom are given together, the involvement of the subcutaneous tissue, despite the absence of artificial hydremia, is very extensive, without, however, the occurrence of increased transudation elsewhere in the body.

From a comparison of the chief experiments and their controls it would appear justifiable to conclude that, for the production of general edema, the three factors—nephritis, vascular injury and an increase of the body fluids—are all essential. No one of these alone, and no combination of two, is sufficient. The frequent occurrence of anuria precludes the possibility of a true hydremia, due to a loss of albumin, being a factor. That cardiac insufficiency may play a part is suggested by the frequent occurrence of edema in dependent parts, but on this point I have been unable to reach a definite opinion.

*Nature of the Kidney Injury.*—In regard to the question of kidney injury, it has seemed advisable to investigate one phase of this influence; that is, whether the part played by nephritis in the production of edema is the result of a change in the character of the renal function or is due to a reduction of functional area. To determine this point, experiments have been made on ten animals, the kidney substance of which had been mechanically reduced by operation. From each the entire kidney and one-half (upper pole) of the left were removed under ether anesthesia according to the method described by Dr. Sampson<sup>18</sup> and myself. Treatment was begun, in order to forestall compensatory hyperplasia, on the day after the operation and was carried on as in the ricin and venom experiments, except that chromate solution was not given for the production of nephritis. Two animals received ricin and venom, respectively: two, water only; two, water and ricin; two, water and venom, and one, water and both ricin and venom. The last of these died on the fourth day, as did also one of those receiving water only (renal hemorrhage). Of the others, one died on the sixth day, and the rest were killed one

18. Sampson (J. A.) and Pearce (R. M.): A study of experimental reduction of kidney tissue with special reference to the changes in that remaining. *Jour. Exper. Med.*, 1908, x, 745.

week after the operation. In none was there evidence of definite edema of the tissues or undue accumulation of fluid in the body cavities, although in the animal receiving venom only and in one of those receiving water only, the serous cavities were unusually moist and contained a few drops of free fluid.

The comparison of these experiments with those in which nephritis was produced indicates that the edema-producing effect of the nephritis is not to be explained by mere diminution of functional area.

#### IV. THE EFFECT OF NEPHROTOXIC IMMUNE SERUM

Heineke's observation, that the serum of uranium nephritis, when injected into a hydremic rabbit poisoned with chrome salts, had the power of causing edema, suggested a study of the influence of nephrotoxic immune serum. The result obtained by Heineke may be explained either by the action of substances which act as lymphagogues of the second order or by the injurious effect of some substance or substances acting on vascular endothelium and causing an alteration in the permeability to fluids. As such substances, developing in the course of nephritis, might be similar in nature to those of a nephrotoxic immune serum, which, as has been shown elsewhere,<sup>19</sup> has other properties than its influence on the kidney, such a serum was prepared. This was done by injecting into small dogs the washed kidneys of rabbits. The serum thus procured was injected into rabbits with chromate nephritis.

Preliminary control experiments were first made with normal dog serum. This was administered to two normal rabbits and to three rabbits with chromate nephritis, and at the same time an excess of water was given. In the normal rabbits no evidence of edema could be found. The following experiment is illustrative:

*Rabbit 20*, weighing 2,540 grams, received daily from Feb. 22 to 28, 1908, inclusive, 100 cubic centimeters of water by stomach tube, and on each of these days 5 to 20 cubic centimeters of normal dog serum, either subcutaneously or intraperitoneally. The animal was killed on the 29th. At autopsy no edema could be found.

Similar results were obtained when the serum was given in doses of 5 cubic centimeters daily in the ear vein.

In the second series of controls, with chromate rabbits, two showed only a local edema at site of the chromate injection. In the third occurred a very striking edema of the mucous membrane of the pelvis of the kidneys, the ureters and the bladder.

19. Pearce (R. M.): Concerning the specificity of the somatogenic cytotoxins. *Jour. Med. Research*, 1904, xxv, 576.

*Rabbit 31*, weighing 1,320 grams, received on March 16, 1908, 0.03 grams and on March 17 0.015 gram of potassium chromate; large amounts of albumin appeared in the urine after the first injection and persisted throughout the experiment. On March 16, 17, 18 and 19, 100 cubic centimeters of water were given by stomach-tube, and, on each of these days, 10 cubic centimeters of normal dog serum in the abdomen or the ear vein, alternately. The animal was killed on the 20th. Autopsy showed a well-marked edema of the mucosa and submucosa of the bladder, pelves of the kidneys and of the ureters, the latter being most markedly involved and appearing as solid gelatinous structures with no visible lumina. The retrosternal and peritracheal tissues were also slightly edematous, but no fluid was found in the cavities of the body and no edema of the subcutaneous tissues except very slight swellings at points of injection.

This very definitely localized edema was due apparently to the well-known toxic action of an alien serum affecting the vessels along the path of elimination, and it was hoped that this toxic action would be augmented by the nephrotoxic immune serum. Such a serum, as is now well known, is not specific in its action; in addition to its nephrotoxic power, it has also hemagglutinative and hemolytic properties, and affects, through these activities, the vessels of various organs. These latter properties were those most desired. Two of three animals receiving this serum developed a most extensive edema, while in the third it was localized to the neighborhood of the chromate injections. The following experiment illustrates the severe type:

*Rabbit 22*, weighing 1,480 grams, received on March 10 and 11, 1908, 0.03 gram potassium chromate subcutaneously. On each of the following three days the animal received 5 cubic centimeters of nephrotoxic immune serum in the abdominal cavity and 100 cubic centimeters of water by stomach-tube. The urine, from the 11th on, contained large amounts of albumin and the animal died on the 14th. The autopsy showed a very marked subcutaneous edema over the entire abdomen and thorax which involved all four legs to the paws. The thoracic and abdominal cavities were about half full of clear fluid and the retrosternal and mediastinal tissues were edematous. The pericardial cavity contained an excess of fluid. The kidneys and their pelves and the ureters were greatly swollen by fluid, as were also the tissues about the spleen. The bladder contained no urine.

To control the action of the nephrotoxic serum in absence of chromate nephritis, this serum was injected into two normal rabbits, which received an excess of water, with no results, except a moderate but very definite edema of the retrosternal tissues and with, in one, a small amount of fluid in the pleural cavities. One of these experiments follows:

*Rabbit 25*, weighing 1,630 grams, was isolated on Mar. 9, 1908. The urine was free of albumin on March 10. On March 11 to 15 inclusive the animal received daily 5 cubic centimeters of nephrotoxic immune serum in the peritoneal cavity and 100 cubic centimeters of water in the stomach. On the 12th considerable albumin appeared in the urine and persisted throughout the experiment. The rabbit was killed on the 16th. A peculiar gelatinous edema of the retrosternal tissues was present but no edema of the subcutaneous tissues or accumulations of fluid in the cavities of the body.

From this group of experiments it is evident that, even in the presence of hydremia, the toxic power of normal dog serum is not sufficient to produce serious disturbance, but that in an animal with kidney insufficiency due to a chromate nephritis a definite edema occurs. A nephrotoxic immune serum, which causes serious kidney injury, gives a somewhat similar grade of edema in hydremic animals; but in neither is the edema as severe as in hydremic chromate animals receiving nephrotoxic immune serum. The influence of the latter combination shows the great importance of renal insufficiency; the kidney receives an injury from two different renal poisons, either of which alone is capable of causing a serious nephritis and which combined, in the presence of hydremia, cause an edema as severe as any noted in the course of this investigation.

In these experiments an action on the endothelium of the vessels can not be definitely proved, but from our knowledge of cytotoxic immune sera it is proper to assume that the nephrotoxic serum contains endotheliotoxic as well as hemagglutinative and hemolytic bodies and that these endotheliotoxic substances may have been responsible for the severe type of edema.

An attempt has been made, in connection with this last type of experiment to produce, by similar means, an edema in dogs, but without the administration of an excess of water. Nephrotoxic immune serum, as well as serum from animals with spontaneous and uranium nephritis, has been injected into dogs suffering from chromate nephritis. In these experiments the animal was allowed to drink water freely, but no water was given by stomach-tube. In none of six experiments was edema found.

#### CONCLUSIONS

In uranium nephritis the ease with which edema is produced when an excess of water is administered illustrates the importance of hydremia in the causation of edema. The influence of vascular injury is not very plain in the uranium experiments, but is conclusively shown in those in which ricin, venom and arsenic were administered in the course of a chromate nephritis; in all of these the influence of hydremia is also evident.

A consideration of all the results recorded appears to demonstrate conclusively that, under the conditions of these experiments, plethoric hydremia and vascular injury have equal value with nephritis in the production of edema, and that no one of these three factors acting alone, and no combination of two acting together, is sufficient to cause edema.

The experiments with nephrotoxic immune sera point to the possibility that substances, presumably endotheliotoxic and capable by vascular injury of aiding in the production of edema, may be contained in serum.

338 East Twenty-sixth Street.

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AMERICAN MEDICAL ASSOCIATION, ONE HUNDRED AND THREE DEARBORN AVENUE  
CHICAGO.





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## **THE DETERMINATION OF UREA IN URINES.**

**BY P. A. LEVENE AND GUSTAVE M. MEYER.**

**Received May 1, 1909.**

Recent work on protein metabolism has established the fact that the ratio of urea nitrogen to total nitrogen in urine is a variable, depending on many physiological and pathological conditions and, furthermore

that given conditions exert a constant influence on the ratio. Thus Folin<sup>1</sup> has demonstrated that an increased protein intake caused a rise in the ratio, while in fasting the ratio fell to its lowest value. Levene and Kober<sup>2</sup> have demonstrated that in dogs all the nitrogen intake above the starvation requirement is removed in the form of urea. It has, therefore, become important to be able to estimate the urea output with absolute accuracy. To this end several methods have been recommended in recent years.

Of all recent methods the one of Folin<sup>3</sup> has proven the most satisfactory. But even this method is not free of inconveniences and of certain sources of error. This was very correctly pointed out by C. G. L. Wolf and E. Osterberg<sup>4</sup> and also by Ronchèse.<sup>5</sup> However, we wish to make clear that with care, patience and experience it is possible to obtain by Folin's method uniform and satisfactory urea values.

In order to obviate the inconveniences of Folin's process, Benedict and Gephart<sup>6</sup> have proposed a new method for urea estimation. This process was based on the hydrolysis of urea by hydrochloric acid at the temperature of 150°. This process has actually removed the objectionable feature of the Folin method, but, on the other hand, it has accentuated the possible sources of error. It was proven by Wolf and Osterberg and also found in our laboratory that under the conditions indicated by Benedict and Gephart, not only urea, but also uric acid and creatinine suffer hydrolysis, though in minor degree than urea. It was, however, found in our laboratory that results obtained by the method of Benedict and Gephart were satisfactory for urines with a minimal content of uric acid and creatinine, such as dog's urine. On urines of normal man and of dogs in pathological conditions, the method could not be applied without further modification.

Appreciating the great convenience of the method of Benedict and Gephart and at the same time realizing that the principal source of error lay in the uric acid and creatinine, it was natural to attempt a modification of the method consisting in removing from the urine the uric acid and creatinine. We proposed to accomplish this by removing the basic substances of the urine by means of phosphotungstic acid and estimating the urea in the filtrate. This process offers also the advantage that the ammonia value does not enter into the urea value and that therefore the figures obtained for the urea do not depend on possible sources of error in the ammonia determinations.

<sup>1</sup> *Am. J. Physiol.*, **13**, 66 (1905).

<sup>2</sup> *Ibid.*, **23**, 324 (1909).

<sup>3</sup> *Z. Physiol. Chem.*, **36**, 333 (1902).

<sup>4</sup> *THIS JOURNAL*, **31**, 421 (1909)

<sup>5</sup> *Bull. soc. chim.* [4], **3**, 1138 (1908).

*THIS JOURNAL*, **30**, 1760 (1908).

Doubts have been expressed as to the possibility of removing the ammonia completely from the urine by means of phosphotungstic acid.<sup>1</sup> It can be seen from experiments recorded in this paper that this criticism lacks experimental foundation.

In order to test the process the following series of experiments were performed:

1. The estimation of urea in pure urea solutions (Table I).
2. In solutions of urea, uric acid and creatinine in proportion analogous to their occurrence in urine (Table I).
3. In urines to which varying quantities of uric acid were added (Tables II and III).
4. In urines to which varying quantities of ammonia were added (Table IV).
5. A comparative estimation of urea was accomplished by means of the methods of Folin, Benedict and Gephart and by the modification of the latter process (Table V).

TABLE I.—ANALYSIS OF SOLUTIONS OF PURE UREA IN WATER ALONE AND WITH THE ADDITION OF URIC ACID AND CREATININE.

Solution.	Method.	Per cent. nitrogen.
A. Uric acid and creatinine . . . . .	Kjeldahl	0.0392
	B. and G.	0.0070
	P. T.	0.0028
	Kjeldahl on filtrate	0.0056
B. Urea . . . . .	Kjeldahl	0.9120
	B. and G.	0.9156
	P. T.	0.9150
	Kjeldahl on filtrate	0.9160
C. Urea, uric acid and creatinine . .	Kjeldahl	0.9580
	B. and G.	0.9240
	P. T.	0.9160
	Kjeldahl on filtrate	0.9160

TABLE II.—ANALYSIS OF URINE TO WHICH VARYING AMOUNTS OF URIC ACID AND UREA WERE ADDED.

Solution.	Method.	Per cent. nitrogen.	Calculated value.
A. Uric acid . . . . .	Kjeldahl	0.031	...
B. Urea . . . . .	Kjeldahl	1.795	...
1. Urine, 50 cc. . . . .	} Kjeldahl	0.475	...
		0.384	...
Water, 50 cc. . . . .	} P. T.	0.384	...
2. Urine, 25 cc. . . . .	} Kjeldahl	0.492	...
		0.385	0.383
A, 25 cc. . . . .	} P. T.	0.385	0.383
3. Urine, 25 cc. . . . .	} Kjeldahl	0.480	...
		0.384	0.383
A, 12.5 cc.; water, 12.5 cc. }	} P. T.	0.384	0.383
4. Urine, 25 cc. . . . .	} Kjeldahl	1.374	...
		1.288	1.283
B, 25 cc. . . . .	} P. T.	1.288	1.283
5. Urine, 25 cc. . . . .	} Kjeldahl	0.926	...
		0.832	0.835
B, 12.5 cc.; water, 12.5 cc. }	} P. T.	0.832	0.835

<sup>1</sup> *Pflüger's Archiv.*, 43, 31 (1888); *Ibid.*, 44, 77 and 97 (1889).

TABLE III.—ANALYSIS OF URINE TO WHICH VARYING AMOUNTS OF URIC ACID AND UREA WERE ADDED.

Solution.	Method.	Per cent. nitrogen.	Calculated value.
A. Uric acid.....	Kjeldahl	0.031	...
B. Urea .....	Kjeldahl	1.856	...
1. Urine, 50 cc .....	Kjeldahl	0.440	...
Water, 50 cc .....		0.368	...
2. Urine, 50 cc.....	Kjeldahl	0.448	...
A, 12.5 cc.; water, 37.5 cc.		0.368	0.368
3. Urine, 50 cc. ....	Kjeldahl	0.454	..
A, 25 cc.; water, 25 cc.....		0.368	0.368
4. Urine, 50 cc ... .	Kjeldahl	0.461	...
Water, 50 cc.....		0.368	0.368
5. Urine, 50 cc .....	Kjeldahl	0.810	...
B, 12.5 cc.; water, 37.5 cc		0.726	0.738
6. Urine, 50 cc.....	Kjeldahl	1.011	...
B, 25 cc.; water, 25 cc. .		0.901	0.939
7. Urine, 50 cc ....	Kjeldahl	1.358	...
B, 50 cc .....		1.246	0.286

TABLE IV.—ANALYSIS OF URINES TO WHICH VARYING AMOUNTS OF AMMONIUM CHLORIDE WERE ADDED.

Solution.	Method.	Per cent. nitrogen.
A. Ammonium chloride . . . .	Kjeldahl	0.084
1. Urine, 50 cc . . . . .	Kjeldahl	0.440
Water, 50 cc . . . . .		0.018
	P. T.	0.368
2. Urine, 50 cc . . . . .	Kjeldahl	0.484
A, 50 cc. . . . .		0.056
	P. T.	0.368
3. Urine, 50 cc . . . . .	Kjeldahl	0.473
A, 25 cc . . . . .		0.037
Water, 25 cc.....	P. T.	0.368
4. Urine, 50 cc. . . . .	Kjeldahl	0.451
A, 12.5 cc.....		0.028
Water, 37.5 cc.....	P. T.	0.368

Before recording the results we wish to make clear that we do not claim originality for any one step in the proposed method but desire to call the attention of workers to it, for the reason of its convenience and accuracy.

*Experimental.*—The method finally adopted was as follows: 12.5 cc. of urine were pipetted into a 50 cc. graduated flask and 10 per cent. solution of phosphotungstic acid in 10 per cent. sulphuric acid was added slightly in excess of that required to completely precipitate the basic substances. This amount was best determined on a separate sample of urine as recommended by Pflüger and Bohland.<sup>1</sup> Too large an excess

<sup>1</sup> Pflüger's *Archiv.*, 38, 622 (1886).

of phosphotungstic acid should be avoided as is evident from the results recorded in Table IV. After allowing the mixture to stand for 24 hours, 10 per cent. sulphuric acid were added to complete the volume of 50 cc. and the content filtered through a dry filter paper into a dry flask. Two portions of 20 cc. each were measured into Jena glass test tubes and subjected to heating in an autoclave as described by Benedict and Gephart. The contents of each test tube are equivalent to 5 cc. of the urine employed.

TABLE V.—UREA DETERMINATIONS ON THE SAME SAMPLE OF HUMAN URINE BY THE METHODS OF FOLIN, BENEDICT AND GEPHART AND P. T.

Method.	Per cent. nitrogen.	Ratio of total N. to urea N.
Total N, Kjeldahl.....	0.859	....
Urea, Folin.....	0.726	
	0.725	
	0.725	84.5
Urea, B. and G. ....	0.722	
	0.723	84.2
Urea, P. T.....	0.682	
	0.681	
	0.681	
	0.679	
	0.681	
	0.681	79.5

It was invariably noticed that a granular precipitate was present in the test tubes after heating, probably due to the formation of an insoluble ammonium salt of phosphotungstic acid. The contents of the test tube were washed as completely as possible into a distilling flask. The granular precipitate which adhered persistently to the glass was dissolved in a very small quantity (5 to 10 cc.) of 10 per cent. sodium hydroxide and together with the further rinsings immediately added to the main bulk, still acid in the flask. An excess of alkali was now added (40 cc. of 10 per cent. sodium hydroxide) and the ammonia distilled into decinormal acid.

The above method was used in all the experiments about to be described and designated in the tables by the letters P. T.

In Table I are summarized the results of the experiments on the estimation of urea in solutions of pure urea in water and the influence of added uric acid and creatinine on such determinations. Solutions of uric acid and creatinine (uric acid 0.184 gram, creatinine 0.200 gram, water 100 cc.) and of urea (urea 4 grams, water 100 cc.) were made of approximately twice the strength as occur in the urine. From these solutions the following mixtures were prepared for analysis:

- A. 50 cc. uric acid and creatinine solution + 50 cc. water.
- B. 50 cc. urea solution + 50 cc. water.
- C. 50 cc. uric acid and creatinine solution + 50 cc. urea solution.

Analysis of total nitrogen according to Kjeldahl-Gunning, and for urea nitrogen by the Benedict and Gephart and the P. T. methods were carried out in each mixture. Besides these the total nitrogen in the filtrate from the phosphotungstic acid precipitation was determined. The Benedict and Gephart method was modified in so far as 10 per cent. sulphuric acid was substituted for hydrochloric acid in the hydrolysis.

The results obtained indicate that the accuracy of the urea estimation is not impaired by the addition of phosphotungstic acid. Uric acid and creatinine are practically completely precipitated by phosphotungstic acid, and that which remains in solution is not sufficient to appreciably affect the urea values.

In Tables II and III are recorded figures on a mixed sample of human urines to which varying amounts of uric acid and urea had been added. To make the results comparative the urine was diluted one-half with water previous to its analysis and the mixtures with urea and uric acid diluted in the same proportion. It is evident that if the addition of uric acid does not affect the method then the value of urea nitrogen for the mixture should coincide with the figure obtained on the urine diluted one-half with water. The calculated values in the last column are such as would be expected, considering that the increase of total nitrogen is due entirely to added urea when such was added.

The influence of added ammonium chloride is shown in the figures of Table IV. The same urine was used for this experiment as in the previous series reported in Table III. It is evident from the figures that there is no reason to believe that under these conditions ammonia is not completely precipitated by phosphotungstic acid.

Finally the comparative results of urea estimation on one sample of mixed human urine as obtained by the Folin, the Benedict and Gephart and by the P. T. methods are compiled in Table V. We desire to emphasize the fact that the figures of urea by the P. T. method are duplicates of six distinct analyses of the same urine and not six duplicates of one filtrate.

## FURTHER STUDIES ON THE USE OF THE FERMENTATION TUBE IN INTESTINAL BACTERIOLOGY.

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In a previous communication<sup>1</sup> attention was called to useful information derivable from the study of the gas volume and Gram-stained sediments in fermentation tubes inoculated with feces.

Mention was made of the fact that the vast majority of bacterial types composing the fecal flora in health and disease can develop rapidly in these tubes during the first eighteen hours of incubation, and that the resulting growths are, broadly speaking, to be regarded as indices of the *relative viability* of the various components of this fecal flora as it existed at the time the tubes were inoculated. During this incubation period of eighteen hours, the nutrient material derived from the fecal suspension (which was added with the bacteria at the time of inoculation) suffices to enrich the artificial medium in the fermentation tube to such an extent that even those bacteria which grow poorly or even not at all in the artificial media alone (e. g. *B. bifidus*) proliferate rapidly under these special conditions. At the same time the mixed products of bacterial activity (which doubtless are present in relatively concentrated form in the feces) are so considerably diluted by the media in the fermentation tubes that the various types of bacteria, freed from the inhibitory action of these substances, are enabled to vegetate rapidly from the beginning of incubation in an unusually favorable environment.

It was shown, furthermore, that actively vegetating bacterial cells, which are found in the sediments of these fermentation tubes, are much more distinctive, morphologically, than is the

<sup>1</sup> Herter and Kendall: This *Journal*, v., p. 283, 1908.

case in the stools, where these organisms are subjected on the one hand to the deleterious effects of partial desiccation due to withdrawal of water from the fecal masses in the large intestine and on the other to the various products of bacterial activity which become concentrated in the feces.

The result of this exposure to unfavorable conditions is manifested by a depression of bacterial vegetative activity. This modifies the morphology of the fecal flora both by causing a diminution in size in many varieties and well marked changes in the cellular appearances and staining reactions.

In very young children, where the intestinal flora is relatively simple, and composed chiefly of a few dominant types, the study of the phenomena developed in the fermentation tubes (gas-volume and character of the sediment) furnish particularly valuable indications relative to the bacterial conditions obtaining in the intestinal tract.

Escherich,<sup>1</sup> Tissier,<sup>2</sup> Szeğö<sup>3</sup> and others have studied the distribution of bacteria in the alimentary tract and have noticed that in perfectly normal enteric conditions the upper levels are inhabited mainly by facultative aerobic bacteria, while the lower portions of the colon are dominated by anaerobic bacteria. (Escherich<sup>4</sup> referred to the anaerobic bacilli of the bifidus type as "blue colored colon bacilli.")

Repeated examinations of the flora from healthy intestinal contents, both culturally and with the aid of the fermentation tubes, have confirmed these observations. Marked deviations from this norm, most expeditiously detected by the use of fermentation tubes, are readily discernible, and although in the light of the information at present available it is not possible to fully interpret the finer and more subtle phases of these variations, the more general and distinctive changes to which must be attached the most significance can be confidently outlined.

The following observations bear upon certain seeming discrepancies which often are met with in the routine use of the fermenta-

<sup>1</sup> Escherich: *Darmbakterien des Säuglings*, Stuttgart, 1886.

<sup>2</sup> Tissier: *Recherches sur la flore intestinale du nourrisson*, Paris, 1900.

<sup>3</sup> Szeğö: Die Darmmikroben der Säuglinge und Kinder, *Arch. f. Kinderheilk.*, xxii, 1897.

<sup>4</sup> Escherich: *loc. cit.*; also *Jahrb. f. Kinderheilk.*, xlix, p. 138, 1899.

tion tubes in connection with the study of the fecal bacteria. These are commonly of two types:

A. Abnormal gas volume in one or two of the three commonly used sugars;

B. Apparent incompatibility between the gas volume and the character of the bacteria observed in the Gram-stained sediment of the tube.

A. *Abnormal Gas Volume.* Generally speaking the amount of gas produced respectively in dextrose, lactose and saccharose is about the same under normal conditions; that is, one usually finds that the mixed fecal bacteria liberate about the same percentage of gas from each of these carbohydrates during the first eighteen hours of incubation at 37° C.

(If fermentation tubes of different sizes are used for the different sugars, the larger tubes, irrespective of the carbohydrates contained in them, will usually contain greater volumes of gas than will the smaller tubes. This difference is more apparent than real, however. Upon comparing the length of the gas column with the total length of the closed arm of the fermentation tube one will find that in each instance the *total per cent* of gas is very nearly the same irrespective of moderate fluctuations in the relative size of the tubes used for this purpose.)

While the above statements are true for the majority of normal stools, the average of an extensive series of observations shows that a slightly greater volume of gas is actually produced in dextrose than in either lactose or saccharose; the proportion is about 10 to 9, comparing dextrose and lactose directly. Saccharose and lactose, on the contrary, run very nearly the same in this respect.

One fact stands out rather clearly: Lactose is less easily attacked in general by facultative intestinal bacteria and invading organisms than by the obligate intestinal bacteria. Many of the foreign bacteria produce *alkali* in this carbohydrate, while they generate *acid* in dextrose and saccharose under the same conditions, indicating very clearly that in the former case the organism is deriving its oxygen from the air, or at least is unable to obtain it directly from lactose, while in dextrose and saccharose the oxygen may be derived directly through the combustion of the carbohydrates, which are thus shown to be immediately assimilable.

This lack of assimilative power for lactose is even more strikingly illustrated by the growth of certain proteolytic bacteria of the subtilis type in the fermentation tubes. In lactose frequently there is no growth in the closed arm (provided the lactose is not hydrolyzed) and there is formed a heavy pellicle upon the surface of the open arm, together with a moderate turbidity in the bulb. This is a strong indication that the bacillus is obtaining its oxygen from the air rather than from the lactose. In dextrose and saccharose, on the contrary, there is a well marked turbidity in the closed arm and there is no pellicle upon the free surface of the bulb, at least not for forty-eight hours or more. The reaction is usually slightly acid in these sugars, alkaline in lactose.

Having shown that lactose (a carbohydrate peculiar to animals) is in general less readily attacked by facultative and foreign races of bacteria than are dextrose and saccharose (of vegetable origin), and that the obligate intestinal organisms are able to utilize lactose as well as other sugars in their dietary, the significance of abnormal gas volumes becomes more definite.

Broadly speaking the gas volume produced by mixed fecal bacteria depends upon the relative activities of three distinct types of bacteria: the gas-producing type (aërogenic), the acidogenic type (acidophile and coccal forms chiefly), and those bacteria which produce alkali.

The gas-producing bacteria, when they are dominant in the feces (or in pure culture), as a rule produce a volume of gas equal to about 25 or 35 per cent of the total length of the closed arm of the tube. Experiments carried out by the writer have demonstrated that this gas volume may be increased to a considerable degree when these gas-producing bacteria are growing symbiotically with certain strains of alkali-producing bacteria. The latter appear to neutralize the acid produced by the former to a considerable extent, and the period of gas formation is consequently lengthened by this partial removal of acid which impedes the vegetative growth of these organisms.

Not all alkali-producing bacteria can grow symbiotically with the aërogenic bacteria, however. *B. infantilis*,<sup>1</sup> for example, inhibits the growth of certain of the most common gas-producing

<sup>1</sup> Kendall: This *Journal*, v, p. 419, 1909.

bacilli (*B. coli* and *B. aërogenes*) probably through the production of unfavorable metabolic products. This is true in dextrose and in saccharose (provided the strain of colon bacillus is one that produces gas in the latter carbohydrate; it will be remembered that certain strains cannot ferment this carbohydrate and, naturally, cannot be considered in this discussion), but the reverse is true in lactose. In lactose fermentation tubes inoculated with *B. coli* and *B. infantilis*, the gas volume remains essentially the same as that of *B. coli* alone, while *B. infantilis* grows very slowly in this medium, eventually producing a pellicle, but no turbidity. This organism also produces alkali in lactose when it is grown in pure culture.

Those bacteria which produce acid, on the contrary, and no gas (and we are familiar with three well defined types, the coccil forms, including the intestinal streptococci and micrococci, the anaërobic bifid forms, and the facultative aërobic *B. acidophilus* forms) react in a totally different manner with the aërogenic bacilli. These acidogenic organisms augment the amount of acid produced by the aërogenic bacilli of the coli-aërogenes type, and inasmuch as they frequently proliferate rapidly, they inhibit the growth of the gas-forming bacteria. Evidences of this inhibition are frequently seen almost at the beginning of incubation, and a mixed fecal flora which contains these acidogenic forms, either in large numbers, or as varieties which are in an active state of vegetative reproduction, frequently does not generate gas, or at most only small amounts, much less than normal. Stools not infrequently contain acidogenic bacteria of the acidophile type in large numbers, or in a state of active vegetative reproduction. Such stools are usually relatively desiccated, and as a rule specify a relatively long sojourn in the lower portions of the colon. The coli-aërogenes type of bacilli on the contrary find their most suitable environment in the region of the ileocecal valve, the cecum and at particular levels of the small intestine. Hence, when they are carried to the rectal region, they are exposed not only to the products of their own activity produced in the higher levels of the intestinal tract, but also to the products of metabolism of the acidogens in the lower colon as well as to partial desiccation associated with the withdrawal of water from the fecal mass prior to defecation.

The result of this exposure to an unfavorable environment is a depression of the vitality of the colon-aërogenes bacilli.

This depression of vitality is manifested by the inability of these bacteria to grow with their characteristic luxuriance when they are introduced as a part of the fecal flora into fermentation tubes as outlined above. That this lack of development is due to a depression of vitality rather than attributable to actual death is evidenced by the fact that one may easily recover strains of these bacilli if they are grown in non-saccharine media (where the acidogens no longer can produce acid) or by their isolation from plate cultures.

The result of the inability of the colon bacilli to grow in the fermentation tubes under these conditions is a diminution in the amount or even an absence of gas in the closed arm.

To recapitulate: The gas volume is, broadly speaking, the resultant of the combined activities of three types of bacterial action—the aërogenic (brought about usually by *B. coli* and *B. aërogenes*, commonly), the acidogenic (*B. bifidus* and various strains of *B. acidophilus* as well as the intestinal cocci) and the alkali-producing forms.

The gas is chiefly produced by organisms of the colon type. The amount of gas evolved when these bacteria are dominant is about the same as that produced by pure cultures of these organisms.<sup>1</sup>

The limitation of this gas volume is frequently referable to decreased vegetative activity of the colon-aërogenes bacteria rather than to exhaustion of the food supply, as may be shown by diluting cultures in which the evolution of gas has ceased, with sterile water, or, better, sterile physiological salt solution. When this is done a fresh evolution of gas usually will take place.

This fact may be even more clearly brought out by the use of media containing calcium carbonate in addition to the carbohydrate. Protocols of a few typical experiments with the bacteria of normal babies' stools are tabulated. Control experiments to determine the effect of lime on the various types of bac-

<sup>1</sup> Mention has been made of the increased gas volume that is not infrequently noticed when the gas bacillus (*B. perfringens* or *Bact. Welchii*) is present in unusual numbers (Herter and Kendall, *loc. cit.*). In unusual instances *B. vulgaris* (*Proteus vulgaris*) may increase the amount of gas.

teria failed to reveal any selective action favorable to the growth of the aërogenic bacteria, and it is therefore very probable that the neutralization of the acids formed by the colon bacilli and by the acidogenic types of organisms is the most potent factor associated with the increased gas production in carbohydrate-calcium carbonate media. The parallel horizontal columns indicate the amounts of gas (in millimeters) produced respectively in dextrose, lactose and saccharose, both with and without calcium carbonate, using for this purpose fermentation tubes of the regulation type, and the mixed fecal flora of healthy babies.

WITHOUT CaCO <sub>3</sub>			WITH CaCO <sub>3</sub>		
Dextrose	Lactose	Saccharose	Dextrose	Lactose	Saccharose
20	28	28	51	70	65
15	16	17	60	80	72
21	26	21	60	64	55
22	22	23	73	80	63
2	0	3	32	37	35
17	16	16	76	79	74
15	16	15	55	72	60
7	12	8	45	65	48

Not infrequently one sees a decided augmentation of the gas volume in either dextrose, saccharose or both. It is less common in lactose. One frequently finds in such cases a decided increase in the number of alkali-producing bacteria, particularly of the subtiloid type, many strains of which are capable of growing symbiotically with *B. coli*.

One other instance in which the gas volume in dextrose may be markedly increased deserves mention. The writer has found five cases of this kind among young rachitic babies. There was three times as much gas in dextrose as in either lactose or saccharose in three of these cases. Subsequent investigation showed that there were fairly large numbers of typical paratyphoid bacilli in the feces of these children. These bacteria, it is well known, ferment dextrose with the production of gas, but are unable to form gas in either lactose or saccharose. Cultural experiments indicated that *B. coli* was replaced to a considerable extent by

these paratyphoid bacilli. The presence of paratyphoid bacilli was quite unsuspected in these cases and it was only after the first case had been worked out that a similar possibility was suspected in the other four cases. Investigation showed that this supposition was correct.

B. *Apparent Incompatibility between Gas Volume and the Character of the Bacteria in the Gram-stained Sediments.* This phenomenon, most frequently associated with a very small gas production, and the presence of comparatively large numbers of Gram-negative bacilli of the colon-aërogenes types in the Gram-stained sediment, is difficult to explain.

Cultural examination of the bacteria in such fermentation tubes presenting such apparent incompatibilities between gas volume and character of bacteria always show that viable colon bacilli (or *Bact. aërogenes*) are present in at least moderate numbers, and that they may be cultivated without difficulty as soon as they are removed from the direct influence of products of the vital activity of the acidophile types. The latter, it will be remembered, are noteworthy chiefly because they can grow in the presence of acid of sufficient strength to inhibit the vegetative reproduction of other types of bacteria.

The colon bacilli associated with these acidophile bacteria are perfectly typical in every respect, culturally and morphologically, stain in a normal manner and grow luxuriantly under suitable conditions as stated above. They also produce the amount of gas characteristic for these organisms.

Frequently, if one inoculates a second set of fermentation tubes from the sediments of these abnormal types in which there is little or no gas, the gas volume will be greatly increased in amount in the second set and there will be perfect accord between the gas volume and the types of bacteria found in the sediments, namely, a representative number of Gram-negative bacilli of the colon-aërogenes type.

The explanation which seems to be the most logical and which appears to be substantiated by cultural experiments is as follows: In the first place, there is evidence that colon bacilli frequently proliferate in fermentation tubes until a very decided turbidity is observed in both the closed arm and the bulb before gas production becomes apparent, or, in other words, a consider-

able vegetative development precedes visible gas formation. One sees in the sediments of such tubes perfectly staining, large, well developed colon bacilli-grown in a favorable environment, whereas in the feces one finds similar organisms which stain faintly, due doubtless to the unfavorable environment to which the latter bacteria were subjected.

Thus we may state the case as follows: In the feces from which the bacteria in these abnormal fermentation tubes were derived the acidophilic bacteria were either much more numerous than the colon bacilli, or there were present strains which could vegetate much more rapidly in artificial media than these varieties usually met with. (This statement is well substantiated by actual investigation. One has only to isolate a few cultures of acidophilic bacteria to appreciate the differences between various strains with respect to their ability to grow in even the most favorable artificial media. Some of these bacteria grow luxuriantly from the start, while others do not become acclimated until many transfers have been made.)

Almost as soon as the feces are sown into fermentation tubes, the colon bacilli commence to proliferate. At the same time the acidophile bacteria, either because of their unusual numbers, or because they are of such a nature as to grow more rapidly than those ordinarily met with (or owing to a combination of both of these factors) also begin to develop rapidly. The acidophilic bacteria produce acid in considerable amounts, and rapidly. The colon bacilli also produce acid, and the combined acidity is sufficient in a comparatively short time to prevent the further growth of the colon bacilli, and consequently to inhibit the production of gas. The number of organisms which have already grown, however, is sufficient to form a very fair proportion of the total flora of the sediment. As a result one sees the picture described above, namely, the presence of moderate numbers of Gram-negative bacilli, morphologically (and culturally) *B. coli*, associated with a minimal gas volume.

It is possible that there may be other explanations for this phenomenon, but the one presented is substantiated by cultural experiments, in so far as it is possible to imitate artificially the conditions which bring about this state of affairs. It is not practicable, however, to imitate this condition with absolute regular-

ity in artificial media, chiefly because it is not possible to so govern the relative rate of growths of the two types of bacteria that the sequence of events shall be as above outlined.

NOTE: Another bacterial combination, not mentioned above, results in an increased volume of gas in the bioses, particularly saccharose. Some strains of *B. coli* do not produce gas in saccharose, but in the presence of certain kinds of bacteria which secrete invertin, gas is produced, but more slowly than is usually the case. What has actually happened is that the colon bacillus acts upon the dextrose and laevulose which in turn are derived from the saccharose by the inverting action of the associated organism. This may actually be reproduced, for example, by combining certain acidophilic bacteria with *B. coli*. The former grow slowly and produce a certain amount of easily fermentable substances from the saccharose. As soon as this simple sugar is produced, the non-saccharose-fermenting strain of *B. coli* begins to form gas. The amount of gas generated, however, is always less than would be the case with the same organism in dextrose or lactose, or in parallel cultures using colon bacilli fermenting saccharose. This gas production, it should be stated, is not brought about by the action of colon bacilli upon products of the saccharose from sterilization nor by the accidental presence of hexoses in the saccharose as impurities. One finds in control tubes from the same lot of fermentation media (saccharose) that the colon bacillus by itself cannot produce gas, even if the experiment is prolonged a week.

#### SUMMARY.

Generally speaking the gas volume in fermentation tubes inoculated with infantile fecal flora depends upon the relative vegetative activity of three types of bacterial forms: the aërogenic (*B. coli*, *Bact. aërogenes*, rarely *B. paratyphi*), the acidogenic (*B. bifidus*, *B. acidophilus*, coccal forms), and those organisms which produce alkali.

The colon-aërogenes bacilli are the common gas-forming organisms of the mixed fecal flora; if they are not present or if their vitality be depressed, little or no gas will be produced in fermentation tubes inoculated with feces. The amount of gas which these aërogenic bacteria can generate is in general diminished by the unrestrained activity of acidophilic bacteria, simply because these acid-producing bacteria render the medium unfavorable for the continued proliferation of the colon group. On the other hand, certain alkali-producing bacteria which can neutralize the acid produced by either the colon group or the acidophilic

group render the medium more fitted for the development of the colon bacillus, or at least permit these latter bacilli to grow for a longer time, thus resulting in an increase in the amount of gas formed.

Not all alkali-producing bacilli, however, will grow symbiotically with the colon bacillus. Certain strains actually inhibit the vegetative reproduction of the aërogenic bacteria, particularly in dextrose and saccharose.

Lactose, generally speaking, is less readily attacked by the facultative intestinal organisms. This is more marked in the case of the so-called "wild races," many of which actually produce alkali in lactose, although they generate small amounts of acid in dextrose and saccharose (thus indicating that the lactose cannot readily form a part of their nutriment). In lactose, consequently, the restraining action of these bacteria may be less marked than is the case with dextrose or saccharose. This does not, however, always result in the production of a greater volume of gas in lactose than is the case with the other two sugars: the gas volume may be greater in dextrose and saccharose, as has been indicated above. The amount of gas produced in lactose is, on the whole, more constant than is the case with either dextrose, or saccharose, and, broadly speaking, the gas volume and the character of the Gram-stained sediments in this carbohydrate are better indices of the relative viability of the *obligate* intestinal flora than are either of the other two carbohydrates.

Having established presumptive explanations, based upon experimental data for these observed irregularities in fermentative action of the mixed fecal flora, it is essential to correlate these findings with their antecedent agents in the intestinal tract.

It will be necessary, in order to present the facts logically, to review briefly the distribution of bacterial types in the alimentary canal.

Broadly speaking, upon a mixed diet the upper levels of the tract—from the lower duodenum to the ileum—are colonized by considerable numbers of bacteria of the subtiloid type—organisms which liquefy gelatin, peptonize casein, do not form gas in carbohydrates, but which may produce small quantities of acid in dextrose and saccharose. The reaction is usually alkaline in lactose. In dogs, with a diet rich in proteids, the habitat of these

organisms is extended downward in the tract to the cecum and the higher levels of the colon, although as a rule they are relatively less abundant in this region than in the small intestine.

Escherich<sup>1</sup> noticed that in dogs with a protein-rich diet there was a decided increase in the liquefying forms occurring in the feces, and his experiments in this direction are substantiated by the data presented above.

Coccal forms are usually most abundant in the region of the jejunum, particularly the lower half, and the ileum.

Aërogenic bacilli, chiefly *B. coli* and its variants, are most numerous from the cecum to the transverse colon, while the acidophiles and anaerobes are most frequently met with in the descending colon and rectum, where the anaërobic conditions are most marked.

A diet rich in protein, particularly meat (in dogs) and relatively poor in easily fermentable carbohydrates is therefore attended by an extension of the habitat of the liquefying bacteria, and usually by an increase of the aërogenic bacilli (*B. coli*) while the growth of acidophilic bacteria is restricted. In such instances there is usually a decided augmentation of the gas volume above that characteristic of a mixed diet: the sediments show increased numbers of subtiloid bacilli<sup>2</sup> and aërogenic organisms, while the acidophiles are relatively poorly represented.

A carbohydrate-rich diet, on the other hand (particularly if the carbohydrates are easily fermentable sugars rather than starches) results in a limitation of the liquefying bacteria, and a more or less marked restriction of the development of the aërogenic forms accompanied by a decided increase in the acidophiles. The gas volume is decreased and in the sediments are seen the acidophilic bacteria prominently represented, while the other types are suppressed to a greater or less extent.

While, in general, the above statements are substantiated by actual experiments, the results may be considerably modified by the length of time during which the intestinal contents sojourn in the various levels of the tract, and in interpreting results one should be fully informed upon this point. If the fecal mass

<sup>1</sup> Escherich: *Jahrb. f. Kinderheilk.*, lii, p. 1, 1900.

<sup>2</sup> Cf. Kendall: *loc. cit.*

passes through the colon rapidly, and is voided promptly, the acidophilic bacteria do not have time to proliferate to such an extent as is the case under normal conditions, and the resulting gas volume may be relatively increased, even upon a diet rich in carbohydrates.

It will not be possible in this paper to go more into detail with reference to the relation of gas volume and character of the sediment to diet. Enough has been said to indicate in a general way the fundamental relations between the character of the intestinal bacteria and the phenomena which they originate in the fermentation tubes.

Remembering that the bacteria from the higher levels of the alimentary canal develop more rapidly as a rule in artificial media (fermentation tubes) than do those of the lower colon and rectum, it will be readily comprehended that those factors which limit the development of the acidophilic bacteria in the alimentary tract will tend to increase the gas volume rapidly, while the restriction of the gas volume, on the other hand, in general depends upon the activity of relatively large numbers of acidogenic forms (because these organisms develop more slowly in artificial media than do those from the higher levels).



## A COMPARATIVE STUDY OF THE DIPLOCOCCI OCCUR- RING IN EPIDEMIC CEREBRO-SPINAL MENIN- GITIS AND POSTERIOR BASIC MENINGITIS.<sup>1</sup>

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### PLATE XXVI.

As long ago as 1898, Still (1) concluded from a study of eight cases of simple, posterior basilar meningitis occurring in infants, that the disease is a sporadic form of epidemic cerebro-spinal meningitis, and that the Gram negative diplococcus found in the meningeal exudate of both forms is identical, such slight differences as greater vitality and lack of virulence being due to natural variation. This view was generally accepted, and the observations made on cases which occurred in this country confirmed Still's findings without adding anything important to our knowledge of the subject. According to the clinical view that posterior basilar meningitis, when not of syphilitic origin, is the chronic stage of an acute cerebro-spinal meningitis (Holt) (2), there seemed no reason to doubt the identity of the causative microorganism in the two affections.

During the past two years, since the recent epidemic of cerebro-spinal meningitis appeared in the British Isles, several observers have claimed that posterior basilar meningitis is a disease due to a specific microorganism definitely and uniformly differing from the meningococcus causing the ordinary epidemic disease, the difference consisting in a total lack of agglutination and opsonin reactions of the diplococcus from cases of posterior basic meningitis with the serum from an epidemic case, and vice versa. Thus in 1907, Houston (3), after studying diplococci isolated from two cases of posterior basilar meningitis at the Great Ormond Street Hospital for Sick Children, which reacted with the blood from these

<sup>1</sup> Received for publication April 1, 1909.

children but not with that from epidemic cases in Belfast, while cocci isolated from the Belfast cases failed to react with the blood from the London cases, concluded that there are two races of meningococci differing in agglutination and opsonic reactions. Houston studied both reactions in the same slide, prepared according to Wright's technique for the study of opsonins, thus using a dilution of one to three. At the onset of meningitis the opsonic index for the meningococcus is low, but it rises gradually. Agglutinins appear about the sixth day, and after that time the two reactions may be studied together.

At the meeting of the British Medical Association held at Sheffield in July, 1908, Houston and Rankin (4) reiterated the view that "Still's disease is due to a meningococcus which has much the same cultural characteristics as the coccus of epidemic cerebro-spinal fever, but differs from it entirely in its opsonic and agglutinating properties." Eve and Clements (5) expressed the same view even more forcibly, stating that the sporadic organism shows little or no tendency to agglutinate or phagocyte in the patient's own blood or in the serum from an epidemic case and will not agglutinate in the Flexner-Jobling antimeningitis serum. Ker (6) was enabled to pick out three cases of post-basis meningitis by means of the agglutination reactions, but one of these cases was very favorably influenced by the Flexner-Jobling anti-serum.

Dr. Flexner having obtained, through the kindness of Dr. Houston, three strains of diplococci from cases of posterior basilar meningitis, kindly gave me the privilege of studying them in comparison with a number of strains from epidemic meningitis cases isolated before and after this study was undertaken. In all, seventeen cultures of diplococci from cases of meningitis were at my disposal. Their histories were as follows:

Two were isolated from typical cases of epidemic meningitis, grown in the laboratory for one and two years.

Two were isolated from typical cases of epidemic meningitis, grown in the laboratory for three months.

Seven were isolated from typical cases of epidemic meningitis, during the course of this work.

One was isolated from the circulating blood of a patient with indefinite meningeal symptoms but without acute meningitis.

Three were obtained from Dr. Houston and regarded as posterior basilar strains.

Two were isolated from typical cases of epidemic meningitis, but were not meningococci.

These cultures were divided into three groups for purposes of study as follows: old, proved strains, recent strains and posterior basilar strains. The two which proved not to be meningococci will be described later.

*Morphology.*—All were biscuit-shaped diplococci lying in single pairs or in larger groups, and never forming chains. They were not motile.

To Gram's stain they reacted negatively in smears from cerebro-spinal fluid, cultures, and in the sections of organs of inoculated animals.

*Biology.*—On sheep's-serum-agar slants the growth was profuse and typical: greyish-white, smooth, moist showing a slightly metallic sheen on drying, and when moist of a tenacious, mucoid consistency on removal with the needle. On human ascitic fluid agar and on glucose agar, the growth was abundant. On plain agar it was very scant, but readily visible. In plain neutral bouillon a slight turbidity appeared within twenty-four hours and a small, flaky precipitate formed in another day; no pellicle formed. The reaction of the bouillon became alkaline. In serum broth the precipitate was more profuse; the reaction also became alkaline. Litmus milk remained unchanged.

The old, the recent and the posterior basilar strains showed no appreciable differences in any of these culture media. It was not possible to tell one strain or group from another. The two strains designated as not meningococcus were very different. They were also Gram negative cocci occurring in pairs, but they were slightly larger and less strictly biscuit-shaped. They grew profusely on plain agar, caused a marked clouding of serum-glucose agar (the albumin precipitation of Libman) and a diffuse cloudiness of plain and serum bouillon with the formation of a thin pellicle and a thick precipitate. Milk remained fluid. In serum-litmus water containing dextrose, coagulation occurred within twenty-four hours; also in galactose. The other sugars remained unchanged. The facts

that they were Gram negative diplococci and had failed to ferment lactose, mannite, saccharose, raffinose and levulose were their only points in common with true meningococci. The latter showed, in their reactions to sugars in the presence of serum-litmus water, the first points of differences encountered among the various strains; but again they were not race but individual variations. Dextrose, for instance, was fermented by all but the oldest strain, which had lost the power it possessed in this direction eighteen months earlier. The next oldest strain fermented neither dextrose nor maltose, though it had produced acid in both a year before. All the other strains, thirteen in number, fermented maltose. One recent strain, three months old, failed to ferment dextrose at any time. The culture isolated from the blood during life formed acid rapidly in both maltose and dextrose, coagulating the former after three weeks, the latter not at all. The three posterior basilar strains fermented maltose and dextrose, one coagulating maltose only, one producing a sufficient amount of acid in both varieties of sugar to cause coagulation, and the third leaving both sugar media fluid. Arkwright (7) mentions the fact that four strains of meningococcus studied by him fermented no sugars, and Wilson (8) observed the same phenomenon with one strain. The duration of laboratory cultivation of these cultures is not stated. Arkwright gives it as his opinion that the sporadic strains diverge more than the epidemic ones do, as regards the sugar reactions. With this view our observations do not agree. Galactose, lactose, mannite, saccharose, raffinose and levulose were not affected by any one of these fifteen strains of meningococci.

*Autolysis* was apparent to a marked degree in stained cover slips from all these meningococcus cultures more than twenty hours old, but it was not possible to pick out any one strain as being definitely more or less resistant than another. Suspension of the fifteen cultures in salt solution kept at 4° C. showed no differences in the amount of autolysis present at the end of one, two and three days. The same was true of a series of suspensions kept at 37° C. Nor did the viability of the several strains differ appreciably on solid or in fluid media. One of the older strains invariably outlived all three of the posterior basic strains when kept in the thermostat (37° C.)

on sheep's-serum-agar with or without calcium carbonate. Twenty-four hours after having been heated in a salt solution suspension for thirty minutes at 65° C. the posterior basic cocci were as well preserved as were the older and the recent strains. Evidently the autolytic ferment (9) was the same in all; at least the effects were indistinguishable.

*Agglutination.*—It is much to be regretted that only one human serum was obtainable for agglutination tests. The early administration of the Flexner-Jobling antimeningitis serum made all other cases under observation valueless for this purpose. The single specimen of human serum came from a seven months' old infant on the second day of an attack of epidemic meningitis, before the injection of the anti-serum. As was to be expected, it gave only negative agglutination reactions with its own strain of meningococci and with the older strains under observation.

The Flexner-Jobling antimeningitis serum remained the only available means for making agglutination tests with these cultures, normal horse serum being used as controls. A recent non-carbolized specimen was obtained. With it, the three posterior basilar strains agglutinated in dilutions of one to twenty only, but five recent epidemic strains did not agglutinate even in so low a dilution. On the other hand, the two oldest strains used for the inoculation of the horses from the first were agglutinated by the serum in dilution as high as one to two hundred, as was the diplococcus isolated from the cerebro-spinal fluid from the case of the infant referred to above, the fluid having been withdrawn within thirty-six hours of the onset of the meningitis and before any serum had been injected. Two facts are brought out by these results: (1) that prolonged artificial cultivation was not the determining factor, and (2) that agglutination reactions with the Flexner-Jobling anti-serum do not serve to differentiate epidemic from posterior basilar strains of meningococci. That all strains of meningococci are not agglutinated in the same dilutions of a serum has been noted by Bruns and Hohn (10). The two non-meningococcus cultures were not agglutinated by this serum in dilutions of one to five, nor was the *Staphylococcus aureus*.

*Phagocytosis.*—The final test which the English observers applied to the differentiation of the two races of meningococci was that of

phagocytosis. In this, as in the agglutination tests, I was handicapped by the lack of human serum from patients and convalescents. The only specimen obtainable (*vide supra*) was taken on the second day of the disease, when its opsonic content was low. The anti-meningitis horse serum remained as in the agglutination tests the only available means of study until the inoculation of monkeys was begun. I am aware, therefore, that these tests are not comparable with those upon which Houston based his conclusions. Nevertheless, they are interesting and suggestive. The results with the monkey's serum are given with the protocols of the cases.

Two parallel sets of experiments were made. In one Wright's technique for the study of opsonins, but with diluted serum according to the method of Klien (11), was used; in the other the technique devised by Neufeld and Hüne (12), also with diluted serum, and applied by Neufeld (13) to the standardization of antimeningitis serum was employed. It is based on the observation that immune serum favors phagocytosis because it contains bacteriotropic antibodies which are thermo-stable. The serum was used in dilutions as high as one to five thousand, and the results with both methods were fairly the same. Human leucocytes were used with the Wright technique, those from the peritoneal cavity of a guinea-pig for the Neufeld method. The diplococcal suspensions required were thick. The most striking result of both methods was the variability in the degree of phagocytosis displayed by the same corpuscles to different strains of meningococcus. A table on page 585 gives the results of the first series, made with the Neufeld method.

By the Neufeld method no actual counts are made, but the amount of phagocytosis is estimated and compared in the series of prepared slides, a strain of diplococcus which phagocytes readily, but not spontaneously, being used as a comparison control throughout all the experiments. The opsonic strength of the serum is measured by the dilution at which specific phagocytosis exceeds that which is spontaneous and present in the salt solution control. In Wright's technique two sets of tubes were prepared, one being incubated one and one-half hours, as in the Neufeld method, the other, fifteen minutes only. In the slides prepared from these tubes the engulfed cocci were counted. The results were similar to those given in the

*One and One-half Hours Incubation.*

Strain.	Dilutions of anti-meningitis serum.						Salt sol. control.
	1-100	1-200	1-500	1-1000	1-2000	1-5000	
Old { 720 MSS.	+++	+++	++	+	+	±	±
	+++	+++	+	+	±	—	+
Recent {	St. Luke's	+++	+++	++	+	±	±
	Lebanon	+++	+++	++	+	±	±
	A. S.	+++	+++	++	+	±	±
	Simons	++	+	—	—	—	±
	Ward	++	+	—	—	—	±
	Fischer	++	—	—	—	—	±
	Johnston	++	—	—	—	—	±
	Price	++	—	—	—	—	±
	T. C.	+	—	—	—	—	±
	Silverman	+	—	—	—	—	±
Post-Basic {	Moseley	++	+	—	—	—	±
	Cooper	++	+	—	—	—	±
	Windsor	++	+	—	—	—	±

above table, showing that one of the recent strains was most susceptible to phagocytosis, two other recent strains and an old strain but slightly less so, while one of the posterior basilar strains was as resistant as two recent cultures, and less so than the other two post-basic strains, which were comparable with five recent strains in susceptibility to phagocytosis in immune serum. Expressed in another way, the opsonic power of this serum was present in higher dilutions for some strains of meningococci than for others, exactly as the agglutinins were present in higher dilutions for some strains than for others. Definite group distinctions are not deducible from these results.

Comparing the results obtained in the specimens incubated fifteen minutes with those incubated one and a half hours, we find that phagocytosis occurs in the same serum dilutions in both, but the number of cocci taken up by the cells increases with the time of exposure allowed them. Thus:

*Serum Dilutions.*

Strains.	Wright's technique, 15' incubation.							Wright's technique, 90' incubation.						
	100	200	500	1,000	2,000	5,000	Salt sol.	100	200	500	1,000	2,000	5,000	Salt sol.
720	4.0	2.5	2.2	1.5	1.0	0.1	0.5	8.0	8.0	3.0	2.5	1.5	1.0	1.0
A.S.	1.9	1.5	1.2	1.0	0.8	0.2	0.2	4.0	3.2	2.5	1.5	1.2	0.3	0.4
Johnston	1.4	0.5	0.1	0.1	0.0	0.0	0.1	2.2	0.6	0.2	0.1	0.1	0.0	0.1
Moseley	2.0	1.4	0.6	0.3	0.2	0.1	0.2	2.9	1.7	1.0	0.4	0.2	0.2	0.5

When normal horse serum was used the control diplococcus ceased to be taken up by the leukocytes in a dilution greater than one in two hundred, while in the immune serum this point was not reached until a dilution of one to two thousand was employed. The phagocytic value of the immune serum for this diplococcus was, therefore, ten times the normal. The results with the other strains in normal horse serum were similar.<sup>2</sup>

The three posterior basilar cultures were inoculated into the spinal canal of monkeys and recovered from them by lumbar puncture. These cultures, after having been passed through an animal, did not agglutinate nor phagocyte to higher degrees than before.

The two non-meningococcus cultures showed no difference in the numbers of organisms taken up by human or guinea-pig leukocytes in the presence of salt solution as compared with antimeningitis horse serum, diluted or in full strength. The phagocytic index of the serum toward the two cultures was practically nil. It has not been possible to identify these two strains of cocci, obtained in pure culture from two different adult cases of apparently typical meningitis. Von Lingelsheim (14) encountered ten varieties of bacteria in his studies with material from patients suspected of having meningitis. These were meningococcus, *Diplococcus crassus*, staphylococcus, streptococcus, *Streptococcus mucosus*, *Diplococcus mucosus*, *Diplococcus pharyngis flavus* II, *Micrococcus cinereus* and Gram negative, plump bacilli. From the nine varieties of cocci enumerated, our strains can be differentiated without much difficulty. From *Diplococcus intracellularis* by its profuse growth on all media, lack of autolysis, fermentation of galactose, and its inability to affect maltose. The cocci under consideration are Gram negative, while *Diplococcus crassus* is still described by von Lingelsheim (14) as being Gram-doubtful; moreover, the latter ferments levulose, saccharose, lactose and maltose, in addition to dextrose and galactose. The Gram positive staphylococcus, streptococcus and *Streptococcus mucosus* need no further differentiation, and the morphology and

<sup>2</sup> Dr. Alice Taylor of London very kindly sent serum from two cases of posterior basilar meningitis. Unfortunately the quantity was very small, and the results of an opsonic test (Wright) with one of the posterior basilar strains was entirely negative when the serum reached us. There was not sufficient serum for study with other diplococci.

capsule of *Diplococcus mucosus* exclude it. *Diplococcus pharyngis flavus* II, as also I and III form a yellow pigment and ferment levulose instead of galactose. *Micrococcus cinereus* does not split any sugar, therein resembling *Micrococcus catarrhalis*, from which its smaller size and less profuse growth serve to differentiate it. That one or more of the diplococci described in the literature as found in air or water may be identical with these cocci found in two specimens of spinal fluid is possible, but the lack of data as to their staining power with Gram's method and their behavior toward the saccharids make it absolutely impossible to give an opinion on the subject. Half an agar culture twenty-four hours old inoculated into the peritoneal cavity of a medium-sized guinea-pig killed the animal in from twenty to thirty hours. At autopsy the point of inoculation was found to be hemorrhagic, the peritoneal cavity contained one or two centimeters of turbid fluid, the omentum was rolled up and hemorrhagic and the liver much congested. The edema about the pancreas, so characteristic of meningococcus infections in these animals, was absent. The pleura and lungs presented nothing abnormal. The cocci were recovered from the peritoneal exudate and from the heart's blood.

#### EXPERIMENTAL MENINGITIS.

The strains of diplococcus from posterior basilar cases were inoculated into the spinal canal of monkeys, in order to test the pathogenicity of these organisms in relation to meningitis, it having been shown that *Diplococcus intracellularis* of epidemic cerebro-spinal meningitis causes the disease experimentally in these animals (15).

*Experiment I: Strain Windsor.*—Two twenty-four hours old sheep-serum-agar slant cultures were suspended in 2 c.c. normal salt solution and inoculated into the lumbar spinal canal of a healthy monkey (*Macacus rhesus*). One hour later the animal seemed ill; in three hours he lay quietly on the floor of his cage, then became restless and evidently very ill. Eighteen hours after inoculation lumbar puncture was performed and a drop of turbid fluid obtained. This showed in films many polymorphonuclear leucocytes, some closely packed with Gram negative diplococci which were swollen and stained poorly. A pure culture of *Diplococcus intracellularis* was grown from this fluid. The animal was still ill and very weak but improved throughout the next twenty-four hours.

Lumbar puncture on the second day withdrew 1 c.c. of turbid fluid, containing many leucocytes, many intracellular diplococci and more extracellular ones than on the first day. No growth resulted from this fluid. On the following day the fluid was almost clear, and no cocci were found in the films. On the fourth day the fluid was quite clear and the monkey seemed well, though weak. Recovery was complete. The phagocytic index of this animal's serum was examined on five days with the following results. (Wright's method, 1½ hours incubation.)

20 hours after inoculation.			2 days.		3 days.		4 days.		9 days.	
Dilution.	(A. S.)		W.	A. S.	W.	A. S.	W.	A. S.	W.	A. S.
	Strain Windsor.	Recent strain.								
100	6.0	3.4	3.0	2.6	1.6	1.7	1.3	1.0	2.3	2.0
200	4.8	3.2	2.5	2.2	1.0	0.9	0.8	0.6	1.8	—
500	3.2	3.1	—	2.0	—	—	—	—	1.0	0.9
1,000	2.8	3.0	2.4	1.5	1.0	—	—	—	0.8	0.6
2,000	2.5	1.5	1.8	1.2	0.8	1.5	—	—	—	—
Control	0.8	0.7	0.8	0.7	0.6	0.4	0.7	0.5	0.6	0.5

It becomes apparent from these results that a recent (epidemic) strain of *Diplococcus intracellularis* was phagocyted in as high dilutions of this animal's serum as was the strain of posterior basilar meningitis used for inoculation. The comparatively high phagocytic index one day after the injection, the short duration of the rise and the rapid fall after the second day are also of interest.

*Experiment II: Strain Cooper.*—(a) Three sheep-serum-agar slants, twenty-four hours old, were suspended in 1.5 c.c. salt solution and injected into lumbar region of spinal canal of a monkey. Within one hour animal seemed ill. Died in seven hours. Autopsy—No purulent exudate visible. Vessels of the pia mater intensely engorged over entire brain and cord. Ventricles not dilated. Brain substance of normal color and consistence. Pia-arachnoid over entire cord and medulla, looks turbid. *Films: Cerebral cortex.*—No leucocytes and no diplococci found. *Medulla*—Many leucocytes, in many of which cocci are contained; cocci also outside the cells; autolysis of cocci marked. *Lumbar and dorsal cord*—Enormous numbers of diplococci, both extra- and intracellular; very large numbers of leucocytes. *Choroid plexus*—Few diplococci within and without leucocytes. *Cultures*—No growth from heart's blood or spleen. Pure growths from medulla, lateral ventricles, and spinal cord. *Microscopic examination of sections*—The pia arachnoid of the spinal cord contains an exudate, in some places quite heavy, consisting of pus cells and fibrin. The exudate covers and surrounds the nerve roots and the intervertebral ganglia. The latter seem not to have been invaded. In a few places, probably corresponding with the lumbar puncture wounds, the dura is infiltrated with pus cells. The number of diplococci in the exudate is very large and they are contained chiefly in the leucocytes. The cerebral meninges also contain leucocytes, fibrin, serum in excess

and extravascular red corpuscles. The blood vessels are widely distended. In the depth of the sulci the cellular exudate is replaced by an inflammatory edema with fibrin formation. Over the cerebellum the leucocytic exudate is heavy; within the cortex the perivascular lymph sheaths contain an excess of polynuclear leucocytes.

(b) Two sheep-serum-agar slants suspended in 1 c.c. of salt solution were inoculated into the lumbar region of the spinal canal. The animal became very sick within two hours and died in nineteen hours. *Autopsy*—In the cervical region of the spinal cord the pia-arachnoid was edematous and hemorrhagic. Over the rest of the brain and cord there was marked congestion, but no visible exudate. *Films and cultures* as in *Experiment (a)*. *Microscopic examination of sections*—The exudate in the spinal meninges is less in quantity than in the preceding monkey and it is almost or entirely leucocytic in character. The exudate in the cerebral meninges is also less and consists of a mixture of polynuclear leucocytes and of red corpuscles. The number of diplococci in the leucocytes in the spinal exudate is large; in the cerebral, small.

(c) One solid culture, twenty-four hours old, was suspended in 1 c.c. of salt solution and injected into the lumbar region of the spinal cord of a monkey. The animal was weak and ill during the night. Lumbar puncture on the following morning withdrew very turbid fluid, showing many leucocytes crowded with diplococci; also some extracellular organisms. On the second morning 0.5 c.c. of turbid fluid was withdrawn, showing numerous leucocytes and large numbers of well staining diplococci. Cultures grew well and were pure. On the third day there was some improvement in the animal's condition. The fluid withdrawn by lumbar puncture was still turbid, containing leucocytes but very few diplococci. The following day no diplococci were found and on the fifth day the spinal fluid was clear. The monkey was then well. Examination of the blood showed that while before inoculation the meningococcus (strain Cooper) was not taken up by the leucocytes in dilutions exceeding 1 to 200, that on the first and second days after injection they were phagocyted in dilutions of 1 to 2,000, on the fourth day to 1 to 1,000, and on the seventh day not over 1 to 200. A recent strain (A.S.) again parallel with the one used for inoculation. In the slides prepared from the serum on the seventh day agglutination of the diplococci was apparent. In the earlier specimens it had been absent.

(d) Two-thirds of one serum-agar-culture was injected into the lumbar region of the spinal canal of a healthy monkey. No symptoms of illness developed, but on the day following the inoculation the spinal fluid was slightly turbid, containing leucocytes with intracellular diplococci in small numbers, and still fewer outside the cells. The cocci grew in pure cultures. The phagocytic index of the serum before the beginning of the experiment was nil in dilutions over 1 to 100. It was apparent in dilutions of 1 to 2,000 after twenty-two hours and had fallen to what it was before inoculation on the fifth day, the recent strain (A.S.) running a parallel curve with the one used for inoculation.

*Experiment III: Strain Moseley*.—One and a half sheep-serum-agar cultures, twenty-four hours old, were injected into the lumbar region of the spinal canal of a monkey. He became ill within two hours, and on the following

morning was very sick, lying on the floor in his cage. Two cubic centimeters of turbid fluid were withdrawn by lumbar puncture and in it many leucocytes containing well staining diplococci were found; a small number of autolyzed diplococci were also present. Cultures from this fluid grew well. On the following morning opisthotonos was marked; fibrillary twitchings and general convulsions were produced by disturbance. The pupils were irregularly contracted and reacted slowly. The animal was perfectly rigid unless disturbed by a touch or a loud noise, when a general convulsion came on. The cerebro-spinal fluid was very turbid, and contained many well-staining diplococci inside leucocytes and outside them as well. On the third morning the monkey was very quiet, lying on his side. No convulsions and no twitchings occurred. The eyes were less irregular. The fluid was still turbid, with many diplococci in the leucocytes. The condition changed but slightly during the following day; the animal made attempts to sit up but soon resumed the recumbent position. On the morning of the fifth day it died. The symptoms of the first days were very much like those of a human patient with meningitis and the clinical picture on the second day was most suggestive. The photograph illustrates the condition at this time.

At the autopsy, encephalitis of the right frontal lobe was found. This portion of the cerebrum was very bright red in color, raised above the level of the adjoining lobes, translucent in appearance, studded with punctate hemorrhages. The layer of gray matter was double the thickness of that in the opposite hemisphere. The remainder of the brain and cord showed a moderate congestion of the vessels of the pia mater. *Cultures* from the cerebrum, cerebellum and spinal cord showed no growth. The microscopic examination of the sections of the spinal cord and brain of this animal indicates that it had recovered almost completely from the meningeal infection and that it succumbed to the cerebral lesion. There are present small remains of the exudate in the meninges consisting of leucocytes and proliferated mononuclear cells.

The chief interest centers in the encephalitis which proved to be an infarction affecting a large part of the hemisphere. The tissue in the infarcted area is necrotic, the contained blood vessels are necrotic, and nuclear staining is generally absent. Around some of the blood vessels, now thrombosed, are collections of leucocytes also undergoing necrosis, and in various parts are punctiform hemorrhages and beginning calcium salt deposits. The membranes over the encephalitic focus are inflamed; they show an accumulation of leucocytes and pus cells. The larger branches of the veins are closed wholly or partially by thrombi containing fibrin and trans-

formed, fused, red corpuscles. In places a heavier exudate lies between the membranes and the encephalitic tissue of which the larger part is composed of proliferated pial cells of large size, which have taken up extravasated red corpuscles that are undergoing decolorization.

This case is of unusual interest because of its typical clinical picture, its prolonged course compared with the cases described by Dr. Flexner in previous experiments, and the exceedingly severe and unusual lesion found at autopsy. It should be added that hemorrhagic encephalitis as a complication of cerebro-spinal meningitis in human beings is of occasional occurrence (16).

#### CONCLUSIONS.

The study carried out and recorded in this paper did not lead to the finding of any reliable criteria of difference between strains of *Diplococcus intracellularis* obtained from typical cases of epidemic meningitis and several cultures obtained from cases of posterior basic meningitis.

The successful experiments made with monkeys show that the diplococcus obtained from cases of posterior basic meningitis is capable of setting up rapidly and acutely fatal forms of meningitis and in producing organic lesions of the cerebral tissues of great severity.

This study would, therefore, suggest that the antimeningitis serum should be as useful in cases of posterior basic meningitis so-called, as it has been in epidemic meningitis, especially if it were employed early in the disease.

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#### EXPLANATION OF PLATE XXVI.

The photograph which was kindly made by Dr. Leaming shows the monkey of Experiment III in the condition of opisthotonos. The hair was cut away from the back of the neck in order to show the degree of curvature and retraction of the head.



FIG. 1.



AN EXPERIMENTAL GLOMERULAR LESION CAUSED  
BY VENOM (CROTALUS ADAMANTEUS)

By RICHARD M. PEARCE, M.D.

## AN EXPERIMENTAL GLOMERULAR LESION CAUSED BY VENOM (CROTALUS ADAMANTEUS).<sup>1</sup>

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### PLATE XXIV.

In the course of a recent study of experimental edema (1), rattlesnake venom in small doses was employed, on account of its well-known hemorrhagic action, to bring about the very necessary factor of general vascular injury. For this purpose it proved most satisfactory, without, as a rule, the production of widespread hemorrhages or serious injury to the kidney. The latter, however, in a few instances, presented a very striking and interesting exudative glomerular lesion without evident alteration of the tubular epithelium. It is upon this observation and the experiments to which it led that the present communication is based.

The lesion in question is of double interest: first to those concerned in the experimental study of nephritis; and secondly, to those who have attempted to differentiate the various toxic bodies of snake venom. The recent renewed interest in the study of nephritis by experimental methods centers mainly in an attempt to distinguish between the disturbance due to tubular and to glomerular injury. The important renal poisons affecting only the glomerulus are limited, practically, to cantharidin and arsenic. The addition to this group of venom, which apparently acts through its endotheliotoxic properties is a matter of no small importance to those interested in experimental renal pathology.

On the other hand the lesion throws considerable light on the action of one of the toxic principles of crotalus venom. It may be recalled that in venom poisoning, experimental or otherwise, hemor-

<sup>1</sup> Aided by a grant from the Rockefeller Institute for Medical Research. Received for publication April 5, 1909.

rhagic manifestations are very constant. Mitchell (2), in his first investigation published in 1861, offered no explanation of this lesion, but in his second communication in 1868 (3), as the result of a series of experiments in which venom was applied directly to the exposed mesentery and its action studied under the microscope, he came to the conclusion that hemorrhage is due to the direct action of venom on the vessel wall. "When the venom passes through the peritoneum, it so affects the walls of the capillaries as to allow of their rupture and of the consequent escape of blood." Later, in a third investigation, in association with Reichert (4), it was demonstrable that the hemorrhage occurred not from the arteries and veins but from the capillaries only. At this time they could not find actual breaks in the capillary wall and describe the escape of blood as apparently a filtering of the blood through the capillary wall.

No further important contribution to this subject was made until 1902 when Flexner and Noguchi (5) published the results of an extensive study of the constitution of snake venom. In addition to the hematotoxic and neurotoxic principles they recognized as the chief toxic constituent of crotalus venom, an endotheliolytic body to which they gave the name "hemorrhagin" and this they considered responsible for the hemorrhages. They found the hemorrhages limited to the capillaries and small veins. Although the extravasation took place through actual rents in the vessel wall, these breaks in continuity were not simple ruptures for they observed microscopically a disappearance as though by solution, of the parts of the wall at the site of hemorrhage. They therefore considered hemorrhagin as a cytolsin for the endothelial cells of blood vessels.

This observation of Flexner and Noguchi has not, as far as I am aware, been confirmed, and when I used venom as a vascular poison to aid in the production of an experimental edema I had no thought of confirming their observation, as I believe I have done, by demonstrating lesions in such an exquisitely delicate endothelial structure as the capillary tuft of the glomerulus.

The glomerular lesion to be described is essentially a vascular lesion, due apparently to a change in the endothelium of the glome-

ular capillaries. It presents two types: the first, extensive hemorrhage into the tuft leading to enlargement and distortion of the tuft with or without rupture; the second, an extensive exudation of the fluid or cellular elements of the blood into the tuft or the capsular space. The cells lining Bowman's capsule show no change while the tubular epithelium generally is either normal or shows only a moderate granular or vacuolar degeneration.

A thorough search of the available literature dealing with venom intoxication fails to reveal an earlier study of these lesions. Several investigators, as first Mitchell, speak of hemorrhages in the kidney visible to the naked eye, or of bloody urine in the bladder, and Mueller (6) describes hematuria in three individuals bitten by the tiger snake, but histologic studies of the kidney appear to be lacking.

The experiments number twenty-one, in all of which the rabbit was used. For the most part the animals received venom alone but a few experiments are included from the earlier work on edema and in these an excess of water was administered by the stomach or chrome salts were given subcutaneously.

The venom used was the dried venom of *Crotalus adamanteus* obtained through the courtesy of Dr. Hideyo Noguchi of the Rockefeller Institute for Medical Research. A standard solution was made with 0.85 per cent. salt solution of such strength that 1 c.c. contained 0.5 mg. of the dried venom. A single dose of 0.5 mg. given intravenously is frequently sufficient to cause the death of a rabbit weighing 1,500 gm. in from five to six days, but in such animals I have not found frank glomerular lesions. These occur more frequently when this dose is given on each of two or three days and almost constantly follow a single dose of 1 mg. or a total administration of 2 mg. in four doses. With a large single dose the animal usually develops hematuria or hemoglobinuria within twenty-four hours, but this rarely occurs with two or three small doses. With the latter dosage, albuminuria of varying grade develops, though generally not until late in the intoxication. As a rule, the animals have been killed when a frank hematuria or albuminuria appeared. This was done, with few exceptions, in order to avoid the rapid post-mortem decomposition, characteristic of venom poisoning, so inimical to proper histological study. As

a result, eleven of the fourteen rabbits with renal injury represent lesions of the fifth or sixth day; the other three represent periods varying from one to four days. The seven rabbits which showed no lesions were animals receiving a single dose or several small doses, and, with the exception of one dying in twenty-four hours and one killed after thirteen days, fall within the three to five day period.

The lesions in the fourteen animals with glomerular involvement may, in a general way, be divided into two groups, one in which exudative phenomena predominate and one in which the characteristic lesion is a hemorrhage into the glomerular tuft. The two lesions may be associated in the same kidney though one or the other type usually predominates in any one kidney. Several factors, as the dose of venom, the period of intoxication, the age (weight) of the animal and the simultaneous administration of other substances as chrome salts or excess of water have all been considered in an effort to determine the cause of difference in type. None of these factors appear to have a determining influence. It is noteworthy, however, that glomerular hemorrhages were very prominent in four rabbits receiving potassium chromate in addition to venom. This salt given alone produces no change in the glomeruli which can be recognized anatomically but does cause widespread injury, with extensive necrosis, of the tubular epithelium. That it may in these experiments have influenced the lesion due to venom is possible but the manner of so acting is not clear.

The material for histological study was hardened in Zenker's fluid and in alcohol, imbedded in celloidin and stained with hematoxylin and eosin, Weigert's fibrin stain and Mallory's connective tissue stain. Frozen sections of formalin-hardened material were stained by Sudan III and hematoxylin.

The hemorrhagic lesion will be described first. Its chief feature is the amount of hemorrhage which may occur in the substance of the tuft without rupture of the outer wall and the escape of blood into the capsular space (Fig. 1). The hemorrhage which may be single or multiple causes a distention of the tuft to two or three times the normal size. The red cells are closely grouped and form, frequently, more or less hyaline masses in which the individual cells

may not be distinguishable. This appearance may be due in part to the agglutination and fusion of cells, as described by those who have studied the effect of venom on the red cells. Sometimes when the hemorrhage is distinctly central the greatly thinned outer wall of the tuft gives the appearance of a thin-walled cyst containing blood. If the hemorrhage is to one side, the remainder of the glomerulus is compressed into a crescent-like mass and the capillaries of the altered tuft contain little if any blood. Many of the larger hemorrhages give, on account of the compression of the rest of the tuft, the appearance of a hemorrhage into the capsular space, but that this is not the case can always be demonstrated by finding a narrow clear space between the cells of Bowman's capsule and the outer border of the tuft. Small accumulations of fibrin or serum may be associated with the hemorrhage but leucocytes are rarely found.

Frequently associated with this definitely intra-glomerular hemorrhage are other glomeruli with free hemorrhage into Bowman's capsule with (Fig. 2) or without the presence of fibrin. In such instances definite evidence of rupture of the tuft is usually seen, the broken fragment of the destroyed tuft sometimes lying free in the midst of red blood cells or more frequently attached to one side of the capsule. At other times the glomerular space is filled with blood without evidence of the destroyed tuft. The coils of the convoluted tubules in the neighborhood of such glomeruli usually contain a considerable amount of blood.

The extent of the hemorrhagic involvement varies. Sometimes nearly every glomerulus is affected; in other animals the ratio is one to two, three, four or five; rarely it is only one to twelve or twenty according to the severity of the intoxication.

The second type of lesion is that characterized by exudation. This may occur alone or may be associated with intraglomerular hemorrhages. All the elements of the blood may appear in the exudate. Red cells frequently form a large part of the picture, but exudates of serum only (Fig. 3) or of serum and fibrin are frequent. Polymorphonuclear leucocytes are seldom seen except as occasional isolated cells (Fig. 4).

The exudate, no matter what its character, distends the capsular

space to two or three times its normal size; the tuft is usually compressed at one side but may remain in its central position. According to the bulk of the exudate either the latter or the tuft may take a crescentic shape. A common picture is that of a delicate fibrin network in which are enmeshed red blood corpuscles and the granules of coagulated serum. Sometimes the fibrin appears as a dense hyaline mass surrounded by red cells (Fig. 1). Some glomeruli contain red cells only or serum only. The kidneys of one animal showed in practically every capsular space a large crescentic finely granular mass of serum. In another, a closely packed fibrinous exudate, homogeneous in appearance and staining deeply with eosin, occurred in three out of every four glomeruli.

Not infrequently the exudate is within and limited to the capillary tuft. Under such circumstances the tuft is transformed into a cavity with a thin wall, giving a ring-like appearance; the cavity of the ring being filled with serum and fibrin (Fig. 5). More rarely, two or three small cyst-like cavities in the tuft contain serum and fibrin threads. At other times the capillaries of the tuft are indistinguishable on account of the presence of strands of hyaline fibrin which give the appearance of a fibrinoid degeneration of the tuft.

It is evident that these phenomena constitute a vascular nephritis and that the primary injury is one affecting the glomerular capillaries and analogous therefore to the vascular lesions occurring elsewhere in the body in venom poisoning and leading to hemorrhage. The glomerular lesion differs from hemorrhage elsewhere in that the phenomena of exudation are added to those of extravasation, or at least can be more readily demonstrated. The question naturally arises: Does the study of this lesion of the capillaries of the glomerulus throw any light on the question of the mode of extravasation of blood in venom poisoning? Reference has been made to Mitchell's observation that the hemorrhage occurs from the capillaries and to the demonstration by Flexner and Noguchi of an endotheliolytic body, "hemorrhagin," in crotalus venom. If such a body exists and if it is responsible for the exudation of the fluid elements of the blood and the extravasation of red cells, the glomerular capillaries should show evidence of its action, and

for such evidence I have searched. Readily demonstrable are the coarser changes, as distention or rupture of the tuft by the accumulation of the blood or exudate, all of which point to weakening of the vessel wall. By careful study may be found finer changes indicative of definite injury to the capillary walls. These changes are seen more readily in connection with the exudative type of lesion. The walls of the capillaries frequently appear swollen and granular with pycnotic or swollen, poorly stained nuclei which are apparently reduced in number (Fig. 2). In some glomeruli the outer portion of the tuft has no distinct sharp outline but an irregular indistinct frayed appearance, as if undergoing solution or gradual disintegration (Fig. 3). Several loops of dilated but empty capillaries sometimes may be seen, usually at the periphery of the tuft, entirely devoid of nuclei (Fig. 1). Others containing blood have a distended congested appearance with a similar scarcity or entire absence of nuclei. Occasionally in the midst of a mass of exudate may be seen a tuft with nuclei in its central portion but none at the periphery. Such a picture is suggestive of tissue erosion or solution. These changes, which do not occur in the ordinary forms of experimental nephritis, suggest, in the absence of evidence of definite tissue necrosis, a disintegration process analogous to autolysis and to be explained by the action of Flexner and Noguchi's endotheliolytic body, "hemorrhagin." In support of this view of the selective action of venom on the glomerular endothelium is the almost complete absence of injury to the tubular epithelium. Casts, in which the products of hemolysis are mingled with exudate, were abundant, but the coarse granular and epithelial casts of tubular origin were not present. In most of the animals the tubular epithelium showed no change; in a few, it was granular and swollen and of the general character of a well-marked cloudy swelling; occasionally, a vacuolar degeneration was evident but at no time necrosis or nuclear destruction and only rarely was fat demonstrable by special stain (Sudan III). This holds also for the cells lining Bowman's capsule, which was almost always well preserved and presented properly staining nuclei. This observation in itself, considered in connection with the extensive lesions in the almost contiguous tuft, would appear to be conclusive as to the selective action of venom.

## SUMMARY.

The venom of *Crotalus adamanteus* when administered intravenously to rabbits in properly graded doses causes lesions of the glomerulus of the kidney which may be either hemorrhagic or exudative in character. Both types of lesion are usually associated but either one or the other may predominate. The hemorrhagic lesion, which may be confined to the glomerular tuft, or, by rupture of the latter, may involve the capsular space, is a peculiar localization of the hemorrhage so common in other parts of the body in venom intoxication. On the other hand, the exquisite exudative lesion involving usually the capsular space but sometimes limited, as in the hemorrhagic type, to the tuft itself and with little or no tubular injury, constitutes a type of experimental vascular nephritis, hitherto undescribed, which differs widely in its anatomical appearance from that due to arsenic, cantharidin and other vascular poisons.

As the limitation of the lesion to the glomerulus indicates a selective action of the venom, and as the histological changes in the tuft are suggestive of gradual endothelial destruction and solution, the lesion can be explained by the action of the endotheliolytic body of crotalus venom described by Flexner and Noguchi.

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## EXPLANATION OF PLATE XXIV.

FIG. 1. The figure illustrates hemorrhage limited to disintegrated portion of tuft without involvement of the capsular space. It shows also compression of remainder of tuft with dilated capillary loop devoid of endothelial cells. From a rabbit weighing 1,070 gm. which received 0.5 mg. of venom and 0.05 gm. potassium chromate on first and fourth days and was killed on fifth day (albuminuria).

FIG. 2. The figure illustrates hemorrhage into capsular space and presence of large mass of compact fibrin. From a rabbit weighing 1,265 gm. which received 0.5 mg. of venom on first day, 1 mg. on second day and 0.5 mg. on fifth day; killed on sixth day (hematuria).

FIG. 3. The figure illustrates extensive pure serous exudate in capsular space and also the common type of disintegrative lesion of the tuft. From a rabbit weighing 1,260 gm. which received 0.5 mg. venom on the first and third day and 75 c.c. of water by stomach tube daily; killed on fourth day.

FIG. 4. The figure illustrates combined capsular and intra-glomerular lesions with compression of tuft. Intra-glomerular lesion shows maximum accumulation of leucocytes observed in any lesion. From a rabbit weighing 2,010 gm. which received 0.5 mg. on first, second and fifth days and 100 c.c. of water by stomach daily; killed on sixth day.

FIG. 5. The figure illustrates exudative lesion (fibrin and red blood corpuscles) limited to the tuft which, except for compressed portion below, is transformed into a cyst-like body. (From same kidney as Fig. 1.)

All drawings are made from sections stained in hematoxylin and eosin after Zenker hardening and celloidin imbedding ( $\frac{1}{8}$  obj., 4 oc., Voightlaender).

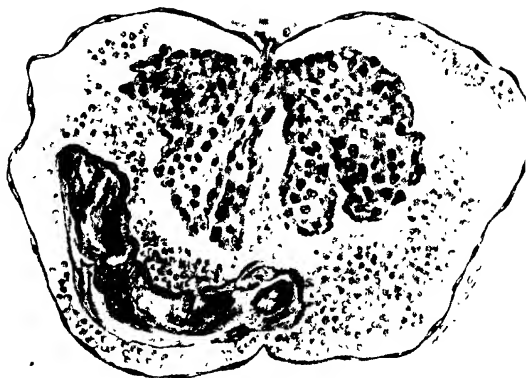


FIG. 2



FIG. 1

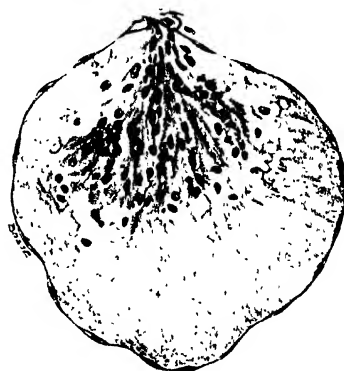


FIG. 3.



FIG. 4.

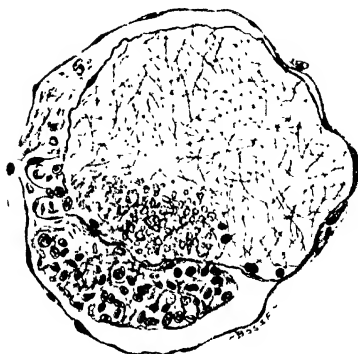


FIG. 5



# STANDARDIZATION OF THE ANTIMENINGITIS SERUM

By JAMES W. JOBLING, M.D.

## STANDARDIZATION OF THE ANTIMENINGITIS SERUM.<sup>1</sup>

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Research, New York.)

The difficulties encountered in standardizing the bactericidal type of immune sera are great and have not yet been successfully mastered. They are especially great in the case of the antimeningitis serum in which bacteriolytic power cannot be utilized as a measure of strength and protective and curative effects, and the experimental *Diplococcus intracellularis* infections in lower animals are also unavailable. The diplococcus of meningitis is not brought to bacteriolysis by the immune serum through amboceptor and complement, and the high variability in pathogenic power of its cultures makes the results of experimental inoculations very untrustworthy. The fatal dose of cultures and of autolyzed toxins fluctuate within wide limits for animals (guinea-pigs) of the same age and weight. The attempt to estimate quantitatively the strength of the antiserum by means of complement deviation cannot be said to have been successful,<sup>2</sup> and in our hands has not yielded accurate or uniform results.

The study of the changes which take place, under the influence of the antiserum, in inoculated animals and in human beings suffering from epidemic cerebro-spinal meningitis indicates unmistakably that the antiserum possesses power to destroy directly the diplococcus, to neutralize a certain amount of the endotoxin yielded by the organism, and above all to bring about an increased phagocytic inclusion and digestion of the diplococcus. The first convincing proof of the manner of action of the antiserum was supplied by the studies of Flexner<sup>3</sup> carried out on monkeys in which an experi-

<sup>1</sup> Received for publication March 1, 1909.

<sup>2</sup> Kolle and Wassermann, *Deut. med. Woch.*, 1907, xxxii, 609.

<sup>3</sup> Flexner and Jobling, *Jour. of Exper. Med.*, 1908, x, 141.

mental cerebro-spinal meningitis had been produced by direct inoculation of the spinal membranes with cultures of *Diplococcus intracellularis*. In brief, Flexner found that in monkeys receiving a lethal dose of the culture an inflammation quickly set in and the diplococci appeared partly free in the fluid exudate and partly within leucocytes quite as in the natural disease in man. In the animals not injected with the antiserum the conditions remained essentially unaltered until death, except that some multiplication of the diplococci sometimes seemed to take place. In the animals receiving the antiserum, the diplococci diminished in number, tended to be entirely taken up by leucocytes, and to suffer rapid degeneration within the latter. These findings led to the view that part of the beneficial action of the antiserum was due to the increased phagocytosis caused by it, a conclusion which has since been verified in the study of many cases of epidemic meningitis in human beings which were treated with the antiserum.<sup>4</sup>

It should be mentioned that Jochmann<sup>5</sup> and Kolle and Wassermann<sup>6</sup> reported uniform results of tests with the antiserum in protecting small animals—mice or guinea-pigs—from the living cultures and thought that use could be made of this method of standardization. But a critical examination of their figures will soon dispel all confidence in its usefulness although they indicate a greater power of the immune serum to protect animals than is possessed by normal serum. A similar conclusion was independently reached by Flexner.<sup>7</sup>

Jochmann,<sup>8</sup> on the other hand, pointed out first that the antiserum possessed greater power of stimulating phagocytosis *in vitro* than normal serum, thus bringing it into line with other immune sera in respect to opsonic properties, but he did not attempt to determine the limits of the opsonic power.

It is difficult to express an opinion on the highly divergent results of Ruppel<sup>9</sup> who claims to have secured a strain of the diplococcus

<sup>4</sup> Flexner and Jobling, *loc. cit.*

<sup>5</sup> Jochmann, *Deut. med. Woch.*, 1907, xxxii, 788.

<sup>6</sup> *Loc. cit.*

<sup>7</sup> Flexner, *Jour. of Exper. Med.*, 1907, ix, 105, 142.

<sup>8</sup> *Loc. cit.*

<sup>9</sup> Ruppel, *Deut. med. Woch.*, 1906, xxxii, 1366.

that is uniformly fatal to mice in doses of 1 cubic centimeter of a 1 to 1,000,000 dilution, and employs this culture in standardizing an antiserum. I believe that in the present state of our knowledge of *Diplococcus intracellularis*, we are justified in viewing Ruppel's claims with suspicion.

Kraus and Doerr<sup>10</sup> are the latest writers to recommend the use of soluble products of the diplococcus, prepared either by Flexner's toluol method<sup>11</sup> or by extraction with N/10 sodium hydrate, for standardization and determination of anti-endotoxic power of the antiserum. The figures given by them do not inspire confidence in the method.

My own experiments with extracts prepared by autolysis and by alkali extraction have been entirely disappointing. In the effort to secure more uniform results the proteids were precipitated from the extracts with alcohol, dried, redissolved in sodium chloride solution, reprecipitated and dried. The resulting powder readily dissolved in water and salt solution but acted irregularly whether administered by subcutaneous intraperitoneal or intracardiac injection in guinea-pigs weighing from 200 to 225 grams, which is the most uniformly susceptible size. The fatal dose varied from 0.001 to 0.01 gram. I am convinced that nothing is to be achieved by this method of standardization.

The methods which have been worked out recently for determining quantitatively the opsonins in serum offer more hope in securing a unit of value for standardization, especially since it has been established that the opsonic power of the antiserum is an important factor in respect to the therapeutic effects. Neufeld<sup>12</sup> is the first person to make quantitative estimations of the opsonic strength of antimeningitis sera and to publish his results. He tested several different samples of antimeningitis sera to determine the greatest dilutions at which they would still cause phagocytosis. The limits of variation were considerable: some samples were active in dilutions of 0.0002 to 0.0005 per cubic centimeter and others only on 0.01 per cubic centimeter.

<sup>10</sup> Kraus and Doerr, *Wien. klin. Woch.*, 1908, xxi, 12.

<sup>11</sup> Flexner, *Jour. of Exper. Med.*, 1907, ix, 105.

<sup>12</sup> Neufeld, *Med. Klin.*, 1908, iv, 1158.

Neufeld is strongly of the opinion that the favorable action of the antimeningitis serum depends largely on its bacteriolytic effect and hence he believes that a measure of the opsonins would also be a measure of its activity and would suffice for standardization. He also admits other effects of the serum including an anti-endotoxic property but he regards them as subordinate to the opsonic property.

I used, at first, in the quantitative study of the opsonins of our antiserum the method recommended by Neufeld, but later I added to it the direct method of counting the included diplococci by a modified Leishman's method. I shall describe the two methods employed in brief and then state the results obtained.

*Neufeld's Method.*—The leucocytes are obtained from the peritoneal cavity of guinea-pigs by injecting aleuronat one day and collecting the exudate the next day. The cells are washed four times in 0.9 per cent. salt solution. After the last washing they are suspended in such a quantity of the saline solution as to equal in opacity a 0.3 per cent. lecithin emulsion in normal saline.

The emulsion of diplococcus is made by adding one cubic centimeter of an equal mixture of bouillon and salt solution to each slanted tube of twenty-four hours' growth of the diplococcus on beef-infusion-glucose agar. This emulsion is quite thick but it is desirable that it should be.

Not every culture of diplococcus intracellularis is suitable for the test. Neufeld laid great stress on the proper selection of the culture and my experience agrees with his. I examined and discarded numerous cultures as being unsuitable either for the reason that they were too readily digested by the leucocytes, or were not readily subject to phagocytic inclusion. Culture No. 720, which was the one selected, fulfilled the conditions very well. As illustrating the great variation in phagocytosis I might mention that certain antisera caused considerable phagocytosis of this culture in dilutions of 1 to 5,000, while with other cultures a corresponding amount of phagocytosis required a dilution of 1 to 500. Culture No. 720 had been under artificial cultivation for more than a year. But the factor of artificial cultivation is not highly important, as I found that certain recently isolated strains were more easily phagocyted than some strains grown in the laboratory for three years.

The tests are conducted in test-tubes of about twelve millimeters in diameter and five centimeters in length. To each tube are added two drops of the diluted antiserum, one drop of emulsion of diplococcus and two drops of suspension of leucocytes. The tubes are incubated at 37° C. for one and one-half hours. At the expiration of the incubation the leucocytes have settled to the bottom of the tubes so that by careful manipulation the supernatant fluid can be poured off, leaving the leucocytes adherent to the bottom of the tube. The spreads are made from the mass of leucocytes and diplococci by placing a platinum loopful on a slide and spreading it with the inclined edge of a second slide. After drying in the air, the spread is fixed for one minute in methyl alcohol and then stained in a 1 to 10 dilution of Manson's methylene blue solution. The control tube contains salt solution instead of antiserum. Where many tests are being carried on at one time a control tube is made for each six or eight of the series. The serum tested ranged in age from ten days to fifteen months. When the sera were less than fifteen days old they were heated at 60° C. for thirty minutes before testing.

The readings are based on the gross appearance of the spreads without counting the phagocytosed diplococci. The spreads made from the tubes containing the higher concentrations of the antiserum showed that very marked phagocytosis had occurred. Many of the leucocytes were completely filled with diplococci; the control spreads would sometimes show leucocytes containing two or three diplococci. In dilutions of the serum at 1 to 1,000 and 1 to 2,000 no difficulty was experienced in interpreting the appearance since the degree of phagocytosis greatly exceeds the controls. When the dilutions exceeded 1 to 2,000 the decision was sometimes less readily made. Whenever doubt existed the tests were repeated several times so as to avoid the experimental error as far as possible.

*Modified Leishman*<sup>13</sup> *Method*.—Feeling that the figures obtained by the Neufeld method were subject to some difference in interpretation, all the antisera were tested as follows: the leucocytes were derived from the circulating blood of the dog, the emulsion of the diplococcus was prepared much thinner, and capillary tubes were used for the mixture to be incubated, after the manner recom-

<sup>13</sup> Leishman, *Brit. Med. Jour.*, 1902, i, 73.

mended by Leishman. The included cocci were counted and the greatest dilution yielding a higher count than the saline control was taken as representing the ultimate strength of the antiserum.

The figures yielded by the two methods are in remarkable agreement so that it will be necessary to give only one table showing the general results obtained. In practice, the second or Leishman method is somewhat more convenient.<sup>14</sup>

The two methods were applied to the antisera obtained by mixing the sera from the three horses used for immunization as is done in preparing the antiserum for distribution. The table gives the numbers of the sera tested, the numbers of the horses and the age of the antisera at the time of the testing.

TABLE I.  
*Opsonic Strength of the Mixed Antisera.*

Number of Serum.	Horses Used.	Age of Serum	Greatest Dilution.
7	Nos. 1 and 2	15 months	1- 800
12	Nos. 1 and 2	13½ "	1- 2,000
17	Nos. 1 and 2	10 "	1- 2,000
19	Nos. 1 and 2	9 "	1- 5,000
20	Nos. 1, 2 and 3	8½ "	1- 2,000
21	Nos. 1, 2 and 3	8 "	1- 2,000
22	Nos. 1, 2 and 3	7½ "	1- 2,000
23	Nos. 1, 2 and 3	7½ "	1- 3,000
24	Nos. 2 and 3	7 "	1- 1,000
25	Nos. 2 and 3	6½ "	1- 1,000
26	Nos. 1 and 2	4 "	1- 1,000
27	Nos. 1, 2 and 3	3½ "	1- 800
28	Nos. 1, 2 and 3	3 "	1- 2,000
29	Nos. 1, 2 and 3	2½ "	1- 5,000
30	Nos. 1, 2 and 3	2 "	1- 5,000
31	Nos. 1, 2 and 3	1½ "	1- 8,000
32	Nos. 1, 2 and 3	15 days	1-10,000
Normal horse serum.		30 and 60 days	1- 80

<sup>14</sup> I am indebted to Dr. R. V. Lamar for a slight modification of the methods which has led to more accurate results. Owing to the readiness with which the diplococcus is dissolved in the leucocytes, it is important to leave them in contact for the briefest possible period. By incubating the serum dilution and diplococci at 37° C. for one hour, before adding the leucocytes, and the mixture of the three components for an additional thirty minutes, maximum phagocytosis is secured and the diplococci are not so acted upon as to lose their staining power.

The variations shown in the table may at first sight appear contradictory, but on analysis they will prove not to be so. In the beginning of the work on the antimeningitis serum, only one horse was used in the preparation of the serum and it was not until several months later that a second horse was subjected to immunization. Sera Nos. 7, 12, 17 and 19 were prepared from a mixture of the sera of horses Nos. 1 and 2. The power rose in this mixed serum from 1 to 800 to 1 to 5,000 in about six months time. Somewhat later a third horse was added, and serum No. 20 is the first lot containing serum from horse No. 3.

The drop in opsonic activity has been attributed to the effect of serum from horse No. 3 that was of low strength. Gradually the strength of the mixture rose to 1 to 3,000 (sample No. 23). Shortly after the preparation of sample No. 23, the horse No. 1 developed fever which continued for several days so that the horse was not bled the next regular time. Hence serum (sample No. 24) was composed of sera from horses Nos. 2 and 3 which had been treated a shorter time than horse No. 1. The drop in opsonic activity is marked, as the limit of the next two samples was only 1 to 1,000. After serum No. 25 was prepared the horses were sent to the farm for the summer—from June until September, during which period they received no inoculation. Samples Nos. 26 and 27 show the falling off due to the summer's rest. Inoculations were resumed and a progressive increase in opsonic strength produced in the combined sera, the highest titre being reached in sample No. 32 in which the limit of dilution was 1 to 10,000. The opsonic value of normal horse serum is low and in the sample recorded was 1 to 80.

TABLE II.  
*Opsonic Strength of the Separate Antisera.*

No. of Serum.	Horse No. 1	Horse No. 2	Horse No. 3.
No. 26, Sept. 19, '08	1- 2,000	1-1,000	1- 200
No. 28, Oct. 16, '08	1- 8,000	1-2,000	1-1,000
No. 29, Oct. 30, '08	1-10,000	1-2,000	1-2,000
No. 32, Jan. 7, '09	1-20,000	1-5,000	1-2,000

Beginning September, 1908, the sera from the several horses were tested separately several times. Table II gives the results of four tests of each horse and exhibits clearly the effects of inoculations

continued over a long period of time. In September, 1908, Horse No. 1 had been under treatment for two years; horse No. 2 for fourteen months; and horse No. 3 for eight months.

The serum sample of September 9 was drawn before inoculation was re-commenced after the summer's rest. Horse No. 3 has always given a smaller yield of serum for the amount of blood withdrawn than either horse No. 1 or No. 2. Horse No. 1 yields the greatest volume of serum of the three horses. Hence in the mixtures, the diluting effects of the sera of horses Nos. 2 and 3 are less than would have been the case had the yield of serum been approximately equal.

#### CONCLUSIONS.

The high variability in infectivity of *Diplococcus intracellularis* makes it impracticable to standardize the antimeningitis serum on the basis of the virulence of the diplococcus.

The irregularity of reaction of small animals to the poison or endotoxin of *Diplococcus intracellularis* makes it impracticable to standardize the antimeningitis serum on the basis of endotoxic value.

The want of uniformity in the complement-binding power of the antimeningitis serum, and the absence of established relation between complement-binding power and therapeutic activity make the employment of this method of standardization impracticable.

The part taken by specific opsonins in promoting recovery from infection with *Diplococcus intracellularis* suggests their employment as a measure of the therapeutic activity of the antiserum. Methods of quantitative estimation of opsonic content of the antimeningitis serum being available, it would seem advantageous to adopt for the present as a standard of value a definite and suitable strength in opsonins of the antimeningitis serum.

As a definite and suitable standard of strength a minimum dilution activity of a 1 to 5,000 dilution of the antiserum is proposed.

Since the immune opsonins of the antimeningitis serum appear to be highly durable under proper conditions of refrigeration of the antiserum, the test proposed will be applicable when the antimeningitis serum shall have become an article of commerce.



## BLOOD-PLATELET AND MEGALOKARYOCYTE REACTIONS IN THE RABBIT.<sup>1</sup>

By C. H. BUNTING.

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To one who has had the opportunity of examining the preparations of Dr. J. H. Wright, the last word seems to have been said on the subject of the origin of the blood-platelets and the function of the bone-marrow giant cell, the megalokaryocyte. Yet his published conclusions, that the blood-platelets are portions of giant cell protoplasm constricted off from projected pseudopodia, seem as yet not to have received general acceptance and confirmation, and the earlier views as to the nature and origin of platelets still dominate medical literature touching upon the subject. In consequence, the following study of the megalokaryocytes and of the platelets is offered. An investigation of the relation of the megalokaryocyte to changes in other bone-marrow elements was in progress at the time of Wright's<sup>2</sup> publication, but its completion has been unavoidably delayed, and in resuming it, the author has directed his attention especially to platelet relations, as a result of Wright's discovery.

In taking up the problem of the origin of the platelet the first question to be settled is, what is a platelet? If one follows the view of Schwalbe,<sup>3</sup> he is obliged at the outset to admit that platelets can have no single mode of origin, for he says: "For the study of platelets there is no more appropriate material than the human thrombus. There one finds all forms of blood-platelets, those with and those without the inner body, platelets that stain *in toto* with hematoxylin and those that stain entirely with eosin, platelets that contain more

<sup>1</sup> Received for publication March 15 1909. The investigations forming the basis of this report were aided by a grant from the Rockefeller Institute for Medical Research.

<sup>2</sup> *Boston Med. and Surg. Jour.*, 1906, cliv, 643.

<sup>3</sup> *Ergebnisse der allg. Pathol.*, 1907, xi, ii, 919.

or less clearly hemoglobin and those without a trace of hemoglobin." Such a collection of cell fragments is surely heterogeneous; yet, except that it probably includes platelets, such a mass bears no relation to the platelet found in the normal circulating blood. It requires but one carefully and quickly made blood smear from human or animal source, stained by Wright's stain, to show that the blood contains no such varied group of bodies. On the other hand, except for red blood cells and leukocytes it contains but a single element, which always stains the same—the body for which the name platelet has been appropriated. The platelet shows constantly a definite and characteristic picture, a sharply outlined light-blue body mass in which lie a variable number of granules staining metachromatically, a purple in which the red predominates. No platelet stains with eosin, none takes a deep methylene blue nuclear stain, none shows a trace of hemoglobin. The platelets vary much in size, but not in staining reactions. If one checks his stained smear by examination of a fresh blood specimen in sodium metaphosphate, sodium citrate or sodium fluoride mixture, he finds just as many delicate, refractile, oval or lenticular bodies as he has stained platelets (within the limits of experimental error). This is the platelet dealt with in the following experiments and not the cell fragments to be found in every disintegrating thrombus mass, described by Schwalbe.

The experiments here reported have had as their object the determination of the relation between changes in the platelets and changes in the other blood elements in the circulation, and further, to ascertain whether or not the platelet changes followed any law comparable to those governing leukocytic or red cell variations. The rabbit has been the animal used in this work, chiefly because of the ease of doing blood counts on it, and also because of the author's familiarity with blood reactions in that animal. In the experiments careful erythrocyte and leukocyte counts have been made and then the platelet count obtained by the indirect method, *i. e.*, by ascertaining their number relative to that of the red blood cells in fresh preparations and checking this result by the relative number in carefully made stained smears. As a counting medium for platelets I have used ten per cent. metaphosphate of soda solu-

tion, 0.5 per cent. sodium citrate, and 0.2 per cent. sodium fluoride, all of which give parallel results in my experience. The first apparently preserves the platelets the best, if a preparation is obtained free from phosphoric acid. Although the solutions have been checked against each other, to avoid a possible source of error, a single solution has been used throughout a series of counts. In obtaining the preparation for counting, the rabbit's ear has been shaved, cleaned with alcohol, a drop of the counting solution transferred to the ear by a platinum loop, and a small vein pricked through the drop. The mixture of blood and counting solution is transferred by the loop to the slide, where it is mixed with more of the solution previously placed in the slide, is covered, ringed with vaseline and immediately counted under high power. The author has found a high power dry lens combination (Zeiss obj. 4 mm. oc. 12) preferable to the oil immersion series, as the adhesion of the oil to the lens and cover glass is apt to produce motion among the cells of the preparation. In the counts here recorded, the number of platelets has been determined from the number observed while counting approximately five hundred red cells.

The experimental error by this method in the author's hands has been found to be less than 50,000 platelets per cu. mm. This may seem a high error, but it is one which is almost negligible, being but about 6 per cent. of the total count in the normal rabbit and an error which would cause but little variation in the curves of platelet changes produced in the experiments. Throughout the experiments painstaking efforts have been made to obtain accuracy in all counts. By this technique, the platelets of the normal rabbit have been found to number between 600,000 and 800,000 per cu. mm. and the majority of the author's series have given a count nearer the upper figure; that is, above 700,000.

If there exists any parental relation between red blood cell and platelet, it seems to the author that this relation should be shown in the circulating blood, either during regeneration of red cells or following their destruction. To determine this, rabbits were subjected to an initial bleeding and blood counts made at intervals following. The reaction is shown by the following experiment:

## RABBIT A-iii (BELGIAN).

April 27, 1908	R.b.c. 5,416,000	w.b.c. 14,750	platelets	730,000
	Bled 30 c.c. from ear vein.			
April 28, 1908	R.b.c. 4,736,000	w.b.c. 12,250	platelets	580,000
April 29, 1908	R.b.c. 4,904,000	w.b.c. 17,250	platelets	940,000
May 1, 1908	R.b.c. 4,760,000	w.b.c. 19,000	platelets	1,080,000
May 5, 1908	R.b.c. 5,368,000	w.b.c. 9,125	platelets	800,000
May 7, 1908	R.b.c. 5,752,000	w.b.c. 13,000	platelets	750,000
	Animal killed.			

A similar reaction is shown by Rabbit A-iv.

## RABBIT A-IV (MIXED WHITE AND BROWN).

Wt. 2,000 gm.

Jan. 7, 1909	R.b.c. 5,280,000	w.b.c. 8,000	platelets	600,000
Jan. 11, 1909	R.b.c. 5,280,000	w.b.c. 8,000	platelets	600,000
	Animal bled 20 c.c. from ear vein.			
Jan. 12, 1909	R.b.c. 3,880,000	w.b.c. 12,000	platelets	220,000
Jan. 14, 1909	R.b.c. 3,456,000	w.b.c. 8,000	platelets	1,000,000
	Animal killed.			

In these two experiments we have a sharp and definite reaction on the part of the platelets which, upon studying the figures, appears to bear no parallel relation to either the red cells or the white cells. In both experiments there is a fall in the platelets following the hemorrhage; then an increase to a maximum well above the normal, and in the animal allowed to live (A-iii) a return to approximately the normal number. This maximum, a thrombocytosis, if it may be so called, has occurred in both cases before there is any marked sign of regeneration of the red cells; and in the second case, while the red cell count is in fact still diminishing. Nor is any relation to the leukocytes to be made out in either case. The more typical leukocytic reaction is shown by Rabbit A-iv, a slight leukocytosis the day following the hemorrhage with a quick return to normal, the leukocytosis occurring while the platelets are at their lowest point. In Rabbit A-iii there was a preëxisting leukocytosis, the result of an epidemic nasal infection going through the rabbit pen, which somewhat disturbs the count.

As it had been noticed in an animal which had received repeated doses of saponin intravenously that there was a high platelet count (1,400,000 per cubic millimeter), it was decided to use this drug to

produce blood destruction—a toxic anemia. The results were somewhat unexpected, for the saponin destroys not only the erythrocytes, but also the platelets, as shown by the following experiments:

## RABBIT S. R. K. (ALBINO).

April 3	R.b.c. 6,696,000	w.b.c. 16,750	platelets	800,000
April 3	Injection of 5 mg. of saponin in ear vein.			
April 4	R.b.c. 3,336,000	w.b.c. 45,000	platelets	120,000
	nucleated r.b.c. 11,000			
April 5	Rabbit died.			

## RABBIT S. R. L. (ALBINO).

April 6	R.b.c. 4,968,000	w.b.c. 16,875	platelets	810,000
April 8	Injection of 4 mg. of saponin in ear vein.			
April 9	R.b.c. 2,880,000	w.b.c. 13,780	platelets	140,000
	nucleated r.b.c. 7,300			
April 11	R.b.c. 3,240,000	w.b.c. 17,125	platelets	110,000
April 15	R.b.c. 3,272,000	w.b.c. 11,375	platelets	230,000
April 19	R.b.c. 3,656,000	w.b.c. 7,000	platelets	680,000
April 23	R.b.c. 4,280,000	w.b.c. 8,000	platelets	1,000,000
April 24	Animal killed.			

It is quite apparent from these experiments that destruction of erythrocytes by saponin does not lead to a direct increase in the platelets, or if it does, that they are subsequently destroyed by the drug, and that the high count of platelets originally noted was the result of regeneration of platelets, as evidenced by the gradual increase in Rabbit S. R. L. A point of extreme interest in the regeneration in this rabbit is the greater length of time required for the platelets to reach their maximum number as compared with the time required for them to reach the same point after hemorrhage (Rabbits A-iii and A-iv)—fifteen days in the first, and three and four days in the last two. So great a difference cannot depend merely upon the greater initial reduction in number of platelets. It must depend, it seems to the author, upon a fact pointed out by him in a previous report,<sup>4</sup> and that is that saponin injected intravenously produces an injury to the bone marrow, causing necrosis of cells and tissue and eventually marked scar-tissue formation in the marrow. Such an injury could then influence the regeneration of the platelets only if they were formed in the marrow.

<sup>4</sup> *Jour. of Exper. Med.*, 1906, viii, 625.

Although no relation could be made out in the experiments between leukocytes and platelets, it seemed necessary to put this to further test and several experiments were performed in which sterile inflammations were produced, by the use of croton oil externally, aleuronat in the peritoneal cavity, and turpentine subcutaneously. The experiments showed uniformly an initial fall in the number of platelets and a secondary rise, where the animal was allowed to live. The following rabbit, in which a relatively large dose of turpentine was injected, producing a most extensive subcutaneous exudate, gave the highest reaction in platelets of the author's series, a reaction verified by repetition of the counts in preparations from different veins in both ears and by the stained smears.

RABBIT A-V (MIXED WHITE AND BROWN).

January 19	R.b.c. 6,280,000	w.b.c. 8,500	platelets 720,000
January 19	7 minims of turpentine injected subcutaneously		
January 20		w.b.c. 2,000	platelets 430,000 <sup>a</sup>
January 21	R.b.c. 6,944,000	w.b.c. 2,125	platelets 430,000
January 23	R.b.c. 6,352,000	w.b.c. 6,000	platelets 720,000
January 25	R.b.c. 6,000,000	w.b.c. 10,700	platelets 1,700,000
January 26	R.b.c. 6,096,000	w.b.c. 16,000	platelets 1,300,000

Animal killed.

It will be noted here that there is a reduction in platelets during the early stage of the inflammation, while there is a corresponding leukopenia and an increase in red cells, due to the withdrawal of serum into the inflammatory exudate. This is followed by a thrombocytosis which reaches its maximum and has begun to recede, while the leukocyte count is still on the increase. There is then no parallelism between the leukocytes and the platelets.

It would seem from the uniformity of the counts of the blood elements in these experiments, which have been made with the greatest care for accuracy, that the platelets follow a curve independent of the other blood elements; and that in their regeneration they follow the general pathological law of a regeneration in excess, giving a thrombocytosis comparable to a leukocytosis, or to the erythrocytosis which may be obtained in an animal with dry tissues,

<sup>a</sup>Ratio of platelets and red cells established. Number estimated from red count of succeeding day.

as the rabbit. This independence and uniformity of reaction leads naturally to the conclusion of an independent source of origin and the unity of the platelet group.

The experiments, in so far as the counts are concerned, give no clue as to the origin of the platelets, unless it be given by the saponin experiments in which, after the marrow injury, the regeneration of the platelets is delayed. The evidence of the source of the bodies must be obtained from morphological study of the tissues of the animals used, and from the study of blood smears, appropriately stained. This evidence has already been furnished by Wright, and the author's results are in general agreement with his findings.

The tissues of the animals used in the experiments already cited have been carefully studied for evidence as to the origin of the platelets. Fixation for the routine hematoxylin and eosin specimens has been made in Zenker's fluid; for the application of Wright's special stain, in warm saturated corrosive sublimate solution. Attention has been given especially to the bone-marrow and the megalokaryocytes. From counts made for me some time previously, by Dr. R. V. Lamar, it was evident that the megalokaryocytes are increased in number in certain conditions, particularly in inflammations and in regeneration after hemorrhage or toxic blood-injury. To determine this point, in reference to the platelet supply, counts have been made on the marrows of the animals used in these experiments, and of others in which the blood condition was known. It is impossible that these counts should have mathematical accuracy, for two reasons: first, because of the irregular distribution of the megalokaryocytes in the marrow, and second, because of the difficulty of establishing a normal count. Yet they have greater value than the mere impression gained from a casual observation of the marrow, that the cells are increased or diminished in number. For all counts, marrow has been taken from the middle third of the femur, and they are reckoned for an area of one square millimeter of a section  $5\mu$  thick. The normal number of megalokaryocytes appears to be approximately 20 per square millimeter, varying a few either above or below this number in different fields. Rabbit A-v, 7 days after a turpentine injection, and with a platelet count of 1,300,000, showed 53 megalokaryocytes per unit area; Rabbit A-iv,

three days after hemorrhage, with a count of 1,000,000 platelets, showed 47; Rabbit S. R. K., one day after a saponin injection, with platelets 120,000, showed 19; Rabbit S. R. L., 15 days after a saponin injection, with platelets 1,000,000, had 31 per unit area. Counts in animals not included in the earlier part of this report are also of interest. In a rabbit twenty-four hours after an infection with *Staphylococcus aureus* megalokaryocytes were 12 per unit area: in a second rabbit twenty-four hours after such an injection they were 33; in a rabbit twenty-four hours after an aleuronat peritonitis they were 25; forty-eight hours after a similar injection in another rabbit they were 47, and in another four days after pleural injection they were 37.

When these results are considered, it is apparent that immediately after the establishment of an inflammatory condition or of a toxic anemia in the animal, there is a reduction in the number of megalokaryocytes in the marrow. This reduction is explained by sections from the lung in these cases. It is a fact of common observation that in every case of sharp leukocytic reaction in man a variable and oftentimes large number of megalokaryocyte nuclei are found in the lung capillaries. This is true also of the rabbit. In every case of leukocytosis, and in the reaction to hemorrhage and ricin, and saponin intoxications, a large number of these nuclei are found wedged into the lung capillaries. At times they may get through the lung and may be found in other organs. The demonstration by Askanazy<sup>6</sup> that in the warm stage the megalokaryocyte possesses active amoeboid motion would indicate that this is an active emigration and not a passive washing out or breaking loose of the cells.

The bone marrow deficiency in giant cells thus produced is followed by an increase in their number. Peculiar complex mitotic figures have been found in megalokaryocytes in all of the regenerating marrows studied. Yet I am not ready to answer the question definitely whether these mitotic figures lead to cell division and to an increase in the number of cells, or merely to an increase in the complexity of the nucleus of the cell. At the present stage of my study of the cell, I am inclined to the latter view largely from my failure to find any evidence of either division of the chromatin

<sup>6</sup>Quoted by Schridde, *Anat. Hefte*, 1907, xxiii, 1.

masses or of the cell, while, on the other hand, there are found in these marrows many giant cells with relatively simple nuclei, even merely bi-lobed nuclei. This suggests that these cells develop from a mononuclear cell, and that the complex nucleus of the adult cell is arrived at by repeated but incomplete mitoses. Nevertheless, the occurrence of mitoses in these cells, following the depletion of the marrow, would indicate a heightened activity of these elements.

It may be objected that mere increase in number of megalokaryocytes is not an indication of increased functional activity on their part, and, further, that their increase in number, occurring simultaneously with an increase in the platelets of the circulation, does not necessarily indicate any relation between the two events. This is admitted. Yet in these marrows we find evidence that the giant cells are active in parting with portions of their protoplasm. In fact, we find that a high percentage of them have either no discernible protoplasm or only a narrow rim with ragged outline. For example, in the rabbit A-iv, in the regeneration following hemorrhage, 46 per cent. of the megalokaryocyte nuclei were either without protoplasm, at least as far as could be made out, or had but a slight ragged rim about them. Likewise in Rabbit A-v, in the regeneration after the turpentine inflammation, there were 38 per cent. of such nuclei. This sign of activity is found also in marrows of normal animals, for it is apparently an indication of the normal function of the megalokaryocyte. For the determination of the manner in which the giant cell loses its protoplasm, and the relation to the platelet, specimens of marrow prepared according to the Wright corrosive-acetone-turpentine method and stained by Wright's stain are very satisfactory, although confirmatory evidence is given by Zenker-hematoxylin-eosin specimens. I have not succeeded in obtaining the characteristic staining of the megalokaryocytes in the marrow of the rabbit by the Wright method after methyl alcohol fixation. With corrosive sublimate fixation, however, the staining is usually successful and the picture is definite. The protoplasm of the cell takes a delicate clear blue tint, and in it are imbedded granules with a metachromatic stain. The granules vary in number and distribution. In some cells they are massed about the nucleus, in others they are more or less evenly distributed,

while in still others they tend to be massed toward the periphery and are more or less clumped. The staining reaction is identical with the staining of the protoplasm and granules of the platelets in the circulation when stained in a blood smear with the Wright stain. This identity of staining reaction in the two elements is particularly well brought out in marrow and lung from the same animal carried through the same fixing and staining solutions. In animals with a high peripheral platelet count, the lung capillaries are crowded with platelets, and the observation of them in this situation is rendered easy by the very thin sections one can obtain of the organ. This establishment of the identity in staining reactions of the protoplasm of the megalokaryocytes and its projected pseudopodia and of the platelets is the most valuable feature of the Wright method.

The author has studied with care a series of marrows prepared both by Wright's method and by the Zenker fixation and has found every evidence of the formation and separation of pseudopodia from the megalokaryocyte. These features are most prominent in active marrows, by which is meant marrows taken from animals while the platelet count was increasing or at its maximum, and they are most evident in marrows fixed as soon as possible after the death of the animal. I can understand Schridde's failure to confirm Wright's findings only from the fact that he used chiefly human material obtained at autopsies performed some time after death. In the rabbit these megalokaryocyte projections or pseudopodia vary much in size. One finds short projections of about the diameter of an ordinary platelet, but more often they are from two to three times that width and of considerable length. One can find also every stage of the constricting off process from its inception to its completion with entire separation of the mass. In the highly active marrows, the pseudopodia are often of considerable size, even approaching half of the cell volume. One remarkable picture was seen in which such a large mass was still connected with the protoplasm surrounding the nucleus by a long drawn-out slender neck, while at its distal end it was breaking up into masses of platelet size. Slender pseudopodia of great length were found in the marrow capillaries, and in one animal similar large masses were found in the lung capillaries, one with an area approximately three times.

that of a red cell and showing a central constriction as if in process of segmentation. This mass was clearly not the result of fusion of platelets, as was shown by the arrangement of the granules in it, by the sharp outline and by the fact that the platelets in this lung specimen existed as clear cut individuals and not as fused masses. It is apparently by the stripping off of such large protoplasmic masses that there is left in the active marrows so high a percentage of almost naked megalokaryocyte nuclei.

These marrow and lung findings correspond well with what has been noted constantly in the fresh and stained preparations of the circulating blood made from the animals used in these experiments. The observation has been that in the animals with high counts after a rapid rise in platelets, the platelets have shown a great variation in size with a tendency to a large average diameter and with the occurrence of some very large forms. A recently examined blood smear from a case of Hodgkin's disease, in which there was an unusual number (1,140,000) of platelets,<sup>7</sup> showed that this variation in size of platelets and the occurrence of large forms is not confined to animals, but may take place also in human beings. This smear was very successfully made from the platelet standpoint and showed them well preserved, with sharp outlines and not fused into masses. The size of the platelets was very variable. Some were long and narrow, others more oval. Two of the long, narrow platelets were fully  $15\mu$  in length, and one oval form noted especially was of similar length and of a diameter almost equal to that of a red cell. From the sharp cut outlines of these platelets and from the distribution of the granules, there was not the slightest possibility that they were masses of fused platelets.

As a result of this study of the platelets and the megalokaryocytes the author feels justified in drawing the following conclusions:

1. The platelets of the circulation form a group of elements of uniform structure and of common origin.
2. In their regeneration after destruction or loss from the circulation they follow the usual pathological law of regeneration in excess, producing a thrombocytosis comparable to a leucocytosis.

<sup>7</sup> The discussion of platelet relations in Hodgkin's disease is reserved for a subsequent report.

3. This curve of regeneration shows no parallelism to that of the erythrocytes or of the leucocytes.
4. Synchronous with or preceding the appearance of an increased number of platelets in the blood stream, the megalokaryocytes of the bone marrow are increased in number.
5. The megalokaryocytes, by separation of pseudopodia of various sizes, become reduced to almost naked nuclear masses.
6. These pseudopodia are identical in staining reactions with the platelets, and do in fact form them by further segmentation.
7. The megalokaryocyte is the only source of the blood platelets..

**CONTINUOUS RESPIRATION WITHOUT RESPIRATORY  
MOVEMENTS**

**By S. J. MELTZER AND JOHN AUER**

# CONTINUOUS RESPIRATION WITHOUT RESPIRATORY MOVEMENTS.<sup>1</sup>

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The object of the function of respiration is to supply the animal with oxygen and to remove carbon dioxide. To attain this object the vertebrates are provided with a complicated mechanism of which the respiratory movements are an essential feature. The respiration appears as a continuous chain of rhythmically recurring cycles, each cycle consisting of two antagonistic movements, one which carries air into the body and the other which assists its removal from the body. When the muscular activity of the body is eliminated by one cause or other and the exchange of the gases is carried on by so-called artificial respiration, again the respiration is rhythmically discontinuous and each cycle is composed of the two antagonistic movements: the inflow of air is carried on rhythmically by some external mechanism, while the return of the air is accomplished during the intermission by the elastic forces of the body. The rhythmic antagonistic movements seem thus to be inseparable from the function of respiration.

In studying recently the nature of the mechanism of the respiration in the presence of a double pneumothorax, while the animal is breathing compressed air by the Brauer method of overpressure, we discovered the fact that under certain conditions respiration can be carried on by continuous inflation of the lungs, and without any normal or artificial rhythmical respiratory movements whatever. This observation was verified by many experiments and we will describe here briefly the essential features of the experiment.

A longitudinal slit is made in the trachea of an anesthetized dog or rabbit and a glass tube introduced down to the tracheal bifurca-

<sup>1</sup> Received for publication June 4, 1909.

tion. The protruding end of the tube is then connected with a pressure bottle by means of a *T*-tube, the opening of the free branch of which is regulated by a screw clamp. The air which streams from the bottle under pressure partly escapes through the free branch of the *T*-tube and partly enters the trachea and reaches the bifurcation from which it returns through the space between tracheal wall and tube and escapes through the slit in the trachea and through mouth and nose. It is essential that the tube should fill out two-thirds of the lumen of the trachea, that the slit in the trachea be not too short and that the pressure of the air which enters the *T*-tube should amount to about fifteen to twenty millimeters of mercury. The pressure within the trachea is of course much lower than that. In the connection between the trachea and the pressure bottle are interpolated a manometer, an ether bottle and a bottle with Ringer's solution to keep the mucous membrane of the trachea moist. The essential point of the arrangement is that air is reaching the bifurcation under pressure and returns through another path than that through which it entered. When the air is thus circulating through the trachea the diaphragm descends, the thorax becomes moderately distended and the respiration mostly becomes very slow. The heart beats also frequently become dangerously slow. This danger, however, is easily obviated by an intravenous injection of one milligram of atropin; in a few seconds the pulse becomes frequent and remains so for many hours. The animal may receive now an intravenous injection of curare sufficient to completely abolish any spontaneous or reflex movements; its life is as safe as under regular artificial respiration. When the anterior thoracic wall is removed, the distended lungs are seen to be immobile while the heart continues to beat with a regular rhythm. If the above described arrangement is carried out properly the lungs retain their pink color, the heart continues to beat regularly and efficiently for many hours and the blood-pressure shows but little variation.

We have observed animals four hours and longer under these normal conditions. If the glass tube within the trachea is a little too wide or too narrow in relation to the lumen of the trachea the lungs acquire easily a slightly cyanotic appearance. But then a disconnection of the tube from the pressure bottle for two

seconds, which means a momentary collapse of the lungs, restores immediately the pink color of the lungs and a repetition of this procedure once every three or four minutes is sufficient to maintain the life of the animal in a satisfactory fashion for many hours, although under these circumstances the blood-pressure is subject to frequent variations.

In another method, the tube which conveys the air to the lungs is short—a regular tracheotomy tube—and is tied in firmly in the upper part of the trachea, while another narrower tube is inserted into the trachea through a narrow opening made at a lower place. This tube reaches the bifurcation and serves for the removal of the air. This method also was found to do satisfactory service. In a third method, a long O'Dwyer tube, bent at right angles, was introduced through the mouth and inserted into the larynx. Through this tube a catheter was pushed into the trachea until it reached the bifurcation. Both tubes were then connected with the pressure bottle in such a manner as to let the air enter through the O'Dwyer tube and escape through the catheter. This arrangement, however, has failed as yet to give uniform results. The method, however, is surely capable of improvement and it is probable that it will finally give satisfactory results.

If the air is made to enter the lungs through a short tracheal tube firmly tied into the trachea, the curarized animals die in a very short time from asphyxia. With this method, the spontaneous respirations of the animals are apparently indispensable for the maintenance of their life. The result is not perceptibly better even if the firmly tied-in tube reaches the bifurcation. The difficulty of this method consists mainly in the fact that the removal of the carbon dioxide has to take place against the stream of the air within the tube; while in our method the removal of the carbon dioxide is rather assisted by this stream of air.

The following three points are the essential factors in the success of our method: (1) The lungs are kept in continuous inspiratory state of distension which facilitates the exchange of the gases. (2) The fresh air reaches the lowest part of the trachea. (3) The air escapes by another path (although also through the trachea) than by the one it enters. Under these conditions the supply of oxygen

and removal of carbon dioxide take place apparently in physiological fashion without the aid of any rhythmic antagonistic movements.

Besides the direct physiological bearing of our experiments on the function of respiration the method is destined to be of methodical service in other physiological investigations, for instance in the study of the heart actions where the movement of the lungs is a disturbing factor. This method might in a certain way offer some advantages over the known methods of Langendorff and of Bock-Hering. Furthermore the method promises to be of practical service in various directions. We shall not omit to refer to two statements in the literature which can be considered as forerunners of our method. In the first place, there is the statement<sup>2</sup> that Hook in 1667 maintained the life of a dog for an hour by continuous inflation of the lungs previously punctured at various places. In the second place, we have to mention Nagel's<sup>3</sup> communication according to which the life of curarized pigeons were maintained by sending a continuous stream of air through the humerus which in birds is connected with the air sacs. In this case the air escaped through the trachea. In both instances the air escaped through the paths opposite to those through which it entered. In our method the air enters and escapes through the trachea, although through the separate paths within it.

<sup>2</sup> Rosenthal, Hermann's Handbuch der Physiologie, 1882, iv, 238.

<sup>3</sup> Nagel, *Centralbl. f. Physiol.*, 1900, xiv, 238.



THE BUTYRIC ACID TEST FOR SYPHILIS IN THE  
DIAGNOSIS OF METASYPHILITIC AND  
OTHER NERVOUS DISORDERS

By HIDEYO NOGUCHI, M.D., AND J. W. MOORE, M.D.

# THE BUTYRIC ACID TEST FOR SYPHILIS IN THE DIAGNOSIS OF METASYPHILITIC AND OTHER NERVOUS DISORDERS.<sup>1</sup>

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General paralysis, although in its clinical aspects a disease that can be frequently diagnosed almost at a glance, is often simulated so closely by other disorders and has so many imitators among the psychoses produced by alcohol, vascular lesions and other causes, that various tests have been devised in recent years for its more certain detection. This paper will be devoted to a consideration of the butyric acid reaction (Noguchi) in relation to the diagnosis of syphilitic affections of the central nervous system, in which class of diseases general paralysis is included.

We have subjected the cerebro-spinal fluid from a series of cases of general paralysis, tabes, cerebral and spinal syphilis and some other mental diseases to this test<sup>2</sup> and at the same time to the complement-fixation test of Wassermann<sup>3</sup> and to cyto-diagnosis according to the method of Ravaut.<sup>4</sup>

<sup>1</sup>Received for publication March 15, 1909.

<sup>2</sup>Noguchi, *Jour. of Exper. Med.*, 1909, xi, 84; *Proc. of the Soc. for Exper. Biol. and Med.*, 1909, vi, 51.

<sup>3</sup>Wassermann and Plaut, *Deutsche med. Woch.*, 1906, xxxii, 1937.

<sup>4</sup>Ravaut, *Ann. de dermat. et de syph.*, 1903, iv, 537.

## THE BUTYRIC ACID TEST.

The cerebro-spinal fluid of syphilitic individuals contains, as does the blood serum, a higher content of globulin and especially of euglobulin than normal cerebro-spinal fluid. It is this increase that makes the butyric acid test applicable to the diagnosis of syphilis.

The mode of applying the test is as follows: one or two parts<sup>5</sup> of spinal fluid<sup>6</sup> are mixed with five parts<sup>7</sup> of 10 per cent. butyric acid solution<sup>8</sup> and are heated over a flame to a brief boiling. One part<sup>9</sup> of normal solution of sodium hydroxide is then added quickly to the heated mixture and the whole is boiled once more for a few seconds. The presence of an increased content of protein in the spinal fluid is indicated by the appearance of a granular or flocculent precipitate which gradually settles under a clear supernatant liquid. The intensity of the reaction varies greatly according to the amount of protein which a given specimen contains, but the granular appearance of the precipitate means a positive reaction for syphilis or parasymphilitic affections.

With normal or non-specific specimens there will be a slight opalescence or sometimes a marked turbidity which, however, does not settle out in several hours or even in twenty-four hours.

The test was applied in this manner to all the samples of cerebro-spinal fluid examined and, at the same time, portions of the samples were tested by means of the Wassermann syphilis test which being now established as indicating syphilitic infection in a high percentage of cases with definite histories must be accepted as giving trustworthy results in cases in which definite histories are not obtainable. In some instances the modification of the Wassermann test introduced by one of us<sup>10</sup> (Noguchi) was used in place of the original method.

The cytological examination, which was the second form of control employed, was that of Ravaut, but with one point of difference,

<sup>5</sup> 0.1 or 0.2 c.c. are sufficient and convenient.

<sup>6</sup> Must not contain blood.

<sup>7</sup> 0.5 c.c. for the quantities above specified.

<sup>8</sup> Best in 0.9 per cent. salt solution.

<sup>9</sup> Namely, 0.1 c.c. in this case.

<sup>10</sup> Noguchi, *Jour. of Exper. Med.*, 1909, xi, 392.

as stated, namely, the use of Ehrlich's triacid stain, which was found very satisfactory. In most of the cases two or more such examinations of the cerebro-spinal fluid were conducted at intervals. It is important to note that in no case did the results of the different examinations vary essentially. The rule adopted by us for the cyto-diagnosis was to regard less than ten cells to a field of the microscope given by a one-sixth lens as negative, more than twelve cells as positive, and ten, eleven and twelve cells to such a field as doubtful. The drop employed came within the limits of a one-third inch objective; but experience enables one to make an accurate calculation even when the drop is less uniform in size or has changed by evaporation. The presence of a small number of red corpuscles does not prevent accurate estimation of the white cells. The modification of the cytological method introduced by Alzheimer<sup>11</sup> was employed in a number of instances but without practical advantages.<sup>12</sup>

Since Nonne and Apelt<sup>13</sup> had already observed an increase in the protein fraction, probably consisting of globulin, in the cerebro-spinal fluid of general paralytics, constituting their so-called "phase I," we applied their method to a large number of cases but with very unsatisfactory results. The poor definition obtained between positive and negative readings made the deduction difficult and questionable.

#### RESULTS IN WHICH THE DIAGNOSIS WAS REASONABLY CERTAIN.

In Table I we have brought together our results in 126 cases whose diagnoses could be made reasonably certain from the symptoms. In a part of the cases the diagnosis was confirmed subsequently by autopsies.

The table brings together the results of the three tests mentioned on several classes of syphilitic affections and on a smaller number of cases of nervous affections in which a syphilitic element could be excluded with a high degree of positiveness. A comparison shows immediately that there is a close correspondence in the results

<sup>11</sup> Alzheimer, *Centralbl. f. Nervenl. und Psychiat.*, 1907, xxx, 449.

<sup>12</sup> The cytological study has been carried out by Dr. Moore at the Pathological Institute of the State Hospitals.

<sup>13</sup> Nonne and Apelt, *Arch. f. Psychiat.*, 1907-8, xliii, 433.

TABLE I.

*Results in Cases in Which the Diagnosis Was Reasonably Certain.*

Cases.	No. of cases.	Butyric acid reaction.			Wassermann reaction.			Cell count.		
		+	-	±	+	-	±	+	-	±
Syphilis										
Secondary stage (without nervous symptoms)	3	3	0	0	0	3	0	0	3	0
Tertiary stage (without nervous symptoms)	1	1	0	0	0	1	0	0	1	0
Cerebral syphilis	3	3	0	0	1	1	1	3	0	0
Spinal syphilis	3	3	0	0	2	1	0	3	0	0
Hereditary syphilis	10	9	0	1	8	2	0			
Metasyphilis										
general paralysis										
cerebral	43	37	4	2	32	6	5	39	2	2
tabetic	17	17	0	0	12	3	2	16	1	0
Tabes	11	11	0	0	6	4	1	11	0	0
	91	84	4	3	61	21	9	72	7	2
Psychoses										
Arterio-sclerotic	3	1	2	0	1	2	0	1	2	0
Traumatic	2	0	2	0	0	2	0	0	2	0
Senile	1	0	1	0	0	1	0	0	1	0
Epileptic	6	0	6	0	0	5	1	0	6	0
Alcoholic	7	0	6	1	3	3	1	0	6	1
Manic-depressive	2	0	2	0	1	1	0	0	2	0
Dementia precox	11	1	10	0	1	8	2	1	10	0
Imbecility	2	0	2	0	0	2	0	0	2	0
	34	2	31	1	6	24	4	2	31	1

obtained. On closer inspection the results of the butyric acid reaction are found to agree more nearly with those of cytodagnosis than does the Wassermann test. The explanation for this may reside in the fact that there sometimes occurs in the cerebro-spinal fluid, as in the blood-serum, substances which are antihemolytic and which, therefore, inhibit hemolysis even when all antigen has been omitted from the mixtures. When the attempt is made to eliminate the action of the antihemolysis by diminishing the quantity of cerebro-spinal fluid in the mixtures, a doubtful reaction may result because of too great dilution of the substances acting as antibody.

It is to be regretted that the number of cases of secondary and tertiary stages is so small. The explanation for this fact is to be found in the difficulty of securing the consent of patients not suffer-

ing from nervous affections to submit to lumbar puncture. The results obtained in the four cases are, however, instructive. It will be observed that all four gave the butyric acid reaction and were negative to the Wassermann test and to cytodagnosis. The explanation of the discrepancy is probably as follows: in active, secondary and tertiary syphilis the increase in globulin of the blood plasma affects also the cerebro-spinal fluid, but the absence of specific lesions of syphilis in the central nervous system determines that the cells are not increased and the antibody on which Wassermann's test depends is not produced *in loco* and does not pass with the fluid in sufficient amount from the blood to give the reaction. Moreover, the discrepancy also indicates that there is no necessary relation between the globulin-content and the Wassermann test. The three cases of cerebral syphilis gave positive results with the butyric acid test and cytodagnosis but remained doubtful to the Wassermann test.

The most striking results are given by the cases of general paralysis of which more than 90 per cent. reacted positively. The cases of the tabetic forms of general paralysis and of simple tabes all gave positive reactions. The close agreement between the butyric acid reaction and cytodagnosis is in contradistinction to the discrepancy between the latter and the Wassermann test.

#### AN ANALYSIS OF THE RESULTS FROM THE STANDPOINT OF SYPHILIS.

In order to study the reactions in relation to metasymphilitic affections, we shall omit those cases with obvious active symptoms of syphilis and disregard all cases in which a reliable history is lacking and the physical examination was insufficient to establish the existence of a previous syphilitic infection. As the majority of the cases comprising our material were derived from an hospital for the insane, only a comparatively small number lend themselves to this consideration. Fifty-nine cases are available for analysis.

Those classed as syphilis + are cases with good histories or unmistakable signs of syphilis or both. Those classed as syphilis — gave intelligent statements, denied the disease and no indication of it could be found.

The results show that cases of general paralysis and tabes with definite histories of a syphilitic infection in early life all yield posi-

TABLE II.

*Analysis of the Reactions With Regard to Syphilis.*

Cases.	No. of cases.	Butyric acid reaction.			Wassermann reaction.			Cell count		
		+	-	*	+	-	*	+	-	*
General paralysis and tabes										
Syphilis +	36	34	1	1	26	8	2	36	0	0
Syphilis -	16	12	3	1	10	3	3	11	3	2
Other diseases										
Syphilis +	1	1	0	0	1	0	0	1	0	0
Syphilis -	12	1	11	0	3	8	1	1	11	0

tive reactions to all the tests in a very high percentage, amounting to 100 per cent. for cytodagnosis, 94 per cent. for the butyric acid test and 72 per cent. for the Wassermann test. It is noteworthy, however, that the same class of diseases affecting individuals without previous histories of a syphilitic infection also give a high, though somewhat lower percentage than the first group. For the butyric acid test, which was the highest, the percentage was 75, for the cell count it was 60 and for the Wassermann test 62.5.

The clinical features of these two groups of cases, namely, the one with definite syphilitic histories and the other without, were not different in any way; moreover, the severity of the symptoms does not always run parallel with the intensity of the reactions. There existed, however, a rough parallelism between the increase of cells and the strength of the butyric acid reaction.

With the cases which were neither paralytic nor tabetic, the results were entirely different. A case of dementia precox with a reliable history of a previous syphilitic infection gave positive reaction to all the tests.

Twelve cases without histories of syphilitic infection were examined with the result that one gave positive reaction to all the tests and two others reacted positively only to the Wassermann, but not to the other two tests. All cases of dementia precox constituting this group reacted negatively to all the tests.

It may be recalled here that Raviart, Breton and Petit<sup>14</sup> obtained about 27 per cent. of positive reactions with the Wassermann test using the blood of cases suffering from dementia precox.

<sup>14</sup> Raviart, Breton and Petit, *Rev. de Méd.*, 1908, xxviii, 840.

## THE BUTYRIC ACID TEST AND POST-MORTEM FINDINGS.

In all, seventeen cases of the series have come to autopsy. Fifteen had given positive findings with the butyric acid test, fourteen being diagnosed as general paralysis and one as cerebral syphilis. Two had given negative results with the acid. The autopsy findings and the results of the test agreed in every case.

In a number of cases the results of the examination of the cerebro-spinal fluid has been the means of establishing an otherwise doubtful diagnosis or of correcting an erroneous one. This is true of cases giving negative as well as positive reactions. In how far this result will be more generally applicable to diagnosis, only a wide series of observations can decide. The outlook seems extremely hopeful.

## THE BUTYRIC REACTION IN GENERAL DISEASES.

It would appear that the butyric acid reaction will come to serve a useful purpose in psychiatric diagnosis. This is the opinion of Mott<sup>15</sup> also, who has already expressed his opinion. It is, however, necessary that the limitation of its applicability should be precisely defined by subjecting the cerebro-spinal fluid from other general diseases to the test. Such a wide examination can alone determine whether the reaction is specific for syphilitic lesions, which at the outset seemed doubtful, or whether the changes in the globulin concentration of the cerebro-spinal fluid occurred in many other diseases than syphilis. It could be predicted, of course, that in all conditions of acute exudative inflammations of the cerebro-spinal meninges, the reaction would be obtained. The change in the character of the fluid and the increase in blood proteins predetermined this result. Table III shows that in all acute inflammatory conditions of the cerebro-spinal meninges the reaction is positive. There is, however, no difficulty whatever in making a diagnosis in this class of cases and they do not, therefore, come into account. The precipitate given with butyric acid is very heavy as would be expected. Even with tubercular meningitis the symptoms and other criteria easily differentiate that condition from syphilitic or meta-syphilitic ones. The cases of hydrocephalus are a little more con-

<sup>15</sup> Mott, *British Med. Jour.*, 1909, i, 454.

fusing, but as only two came under observation and one gave the Wassermann test also, the existence of congenital syphilis should be thought of. The general diseases, among which are typhoid fever and pneumonia, with which meningeal implication may be absent are not attended by changes in the cerebro-spinal fluid that lead to the butyric acid reaction.

TABLE III.  
*The Butyric Acid Reaction in General Diseases.*

Cases.	No. of cases.	Butyric acid reaction.			Wassermann reaction		
		+	-	±	+	-	±
Diseases of the meninges							
Epidemic cerebro-spinal meningitis	14	14	0	0	0	14	0
Pneumococcal meningitis	6	6	0	0	0	6	0
Influenzal "	1	1	0	0	0	1	0
Tubercular "	30	30	0	0	0	30	0
Hydrocephalus externus	2	2	0	0	1	1	0
	53	53	0	0	1	52	0
Diseases without meningeal involvement							
Typhoid fever	1	0	1	0	0	1	0
Pneumonia	4	0	4	0	0	4	0
Pulmonary tuberculosis	1	0	1	0	0	1	0
Enterocolitis	2	0	2	0	0	2	0
Rachitis	1	0	1	0	0	1	0
Uremia	2	0	2	0	0	2	0
Septicemia	1	0	1	0	0	1	0
Miscellaneous without nervous involvement	12	0	11	1	0	10	2
	24	0	23	1	0	22	2

On the basis of these tests, we consider that the butyric acid reaction suffices to distinguish normal from pathological cerebro-spinal fluid and especially from that fluid which has been altered through an increase in its protein constituent. We would, therefore, point out that by its means it may be possible to determine the limits of some ill-defined inflammatory conditions of the meninges, such as, for example, the so-called meningitis serosa, in which condition microorganisms have not yet been demonstrated satisfactorily and the cellular exudations are absent, and yet abnormal serous exudations seem to occur. If these exudations contain an excess of protein constituents as compared with the normal transudate the differ-

ence should be appreciable by this test. Moreover, whether this increase is due to or attended by syphilis could be determined by the Wassermann test or the modification of it recommended by one of us (Noguchi).

We believe, moreover, that in tubercular meningitis the butyric acid reaction will always be positive. In a suspected case, therefore, in which no tubercle bacilli have been found and the tuberculin reaction is unconvincing, this test, if negative, would have diagnostic value. We have applied the test to a small number of doubtful cases of suspected tubercular meningitis. Those giving negative results subsequently proved not to be tubercular.

#### SUMMARY.

1. In the secondary and tertiary stages of syphilis without direct involvement of the nervous system the cerebro-spinal fluid yielded the butyric acid reaction of feeble intensity. The fluid under these conditions gave neither a positive cytodagnosis nor the Wassermann test for syphilis.

2. The cerebro-spinal fluid of cases of hereditary syphilis showed a positive butyric reaction in about 90 per cent. and a positive Wassermann test in about 80 per cent. of the cases examined.

3. The cerebro-spinal fluid of cases of cerebral and spinal syphilis yielded the butyric acid reaction and the cytodagnosis in every instance (100 per cent.) and gave the Wassermann reaction in from 50 to 75 per cent. of those examined.

4. The cerebro-spinal fluid from cases of general paralysis gave positive butyric acid reactions in 90 per cent., positive cell counts in 91 per cent., and positive Wassermann test in 73 per cent. of those examined.

5. The cerebro-spinal fluid from cases of tabes dorsalis gave positive butyric acid reactions and cell counts in 100 per cent. and positive Wassermann test in 53 per cent. of those examined.

6. The cerebro-spinal fluid from other forms of psychosis in which a syphilitic history was not obtained gave positive butyric acid reactions and cell count in 2.8 per cent. and Wassermann test in 13 per cent. of those examined.

7. The cerebro-spinal fluid from cases of acute inflammatory diseases of the meninges always gave a flocculent precipitate with the butyric acid reaction but never gave the Wassermann test.

8. The cerebro-spinal fluid from persons suffering from typhoid fever, pneumonia, pulmonary tuberculosis, etc., in which the meninges are not inflamed, gave neither the butyric acid nor the Wassermann test.

9. We think it justifiable to conclude that the butyric acid reaction affords a ready means of distinguishing normal from pathological cerebro-spinal fluid and will prove to be useful in routine clinical practice, especially in detecting syphilitic disease and in confirming or setting aside certain doubtful diagnoses of syphilitic or metasyphilitic lesions of the central nervous system. The reaction should commend itself as a valuable addition to the Wassermann test, the results of which it is capable of confirming and extending.



# DATA CONCERNING THE ETIOLOGY AND PATHOLOGY OF HEMORRHAGIC NECROSIS OF THE PANCREAS (ACUTE HEMORRHAGIC PANCREATITIS).<sup>1</sup>

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Hemorrhagic pancreatitis was clearly recognized about fifty years ago by Klebs,<sup>2</sup> who believed that the eroding action of the ferments formed by the pancreas was responsible for its occurrence. The classification of acute pancreatitis almost universally adopted, and knowledge of the symptomatology of the various types of inflammation date from the Middleton-Goldsmith lecture of Fitz,<sup>3</sup> delivered before the New York Pathological Society twenty years ago. A certain medico-legal importance has been attached to a disease which may be a cause of sudden unexplained death, but it is perhaps noteworthy that some writers who have emphasized this view have described as hemorrhagic pancreatitis the relatively common post-mortem autolysis which occurs in the pancreas. The cause of the lesion has remained obscure until comparatively recent studies have shown that it can be readily reproduced in lower animals by a variety of means. Human cases have been carefully studied with the aid of these experimental data, with the purpose of defining the cause of the lesion.

It is probable that the physiologist, Claude Bernard,<sup>4</sup> produced the lesion, though he failed to recognize it, years before it had been described in man. It is noteworthy that he injected into the pancreatic duct that fluid, namely, bile, which, as subsequent observations have shown, may cause the lesion in man. In his lectures on experimental physiology, published in 1856, Claude Bernard describes the injection of a mixture of bile and sweet oil into the

<sup>1</sup> Received for publication April 20, 1909.

<sup>2</sup> Handbuch der pathologischen Anatomie, Berlin, 1868, i, 556.

<sup>3</sup> *Med. Rec.*, 1889, xxxv, 197, 225, 253.

<sup>4</sup> *Leçons de physiologie expérimentale*, Paris, 1856, ii, 278.

pancreatic duct of the dog; the animal died after eighteen hours; there was intense peritonitis and the pancreas was red and contained numerous ecchymoses. Subsequent observers have shown that acute hemorrhagic pancreatitis accompanied by disseminated necrosis of fat can be reproduced by similar injection of a variety of substances. The list of substances which have been successfully employed is long, and suggests the possibility that mechanical injury of injection is sufficient; nevertheless, numerous control experiments have demonstrated that various bland substances fail to cause the lesion.

Hlava<sup>5</sup> (1890) first showed that typical acute hemorrhagic pancreatitis with foci of fat necrosis disseminated in the abdominal fat, can be produced in animals by injecting gastric juice through the opened duodenum into the pancreatic duct. Bile (Opie)<sup>6</sup> injected into the duct causes the change; the bile salts (Flexner)<sup>7</sup> are effective, but other constituents of bile fail to produce it. Duodenal contents have been subsequently employed with the same result (Polya).<sup>8</sup> The lesion may be produced with weak solutions of a variety of acids including hydrochloric, nitric and chromic acids, and an alkali, sodium hydroxide, has the same effect (Flexner)<sup>9</sup>; injection of formalin causes the lesion. Sweet oil has the same effect; fatty acids and sodium soaps of these acids have been successfully used, but glycerin is ineffective (Hess).<sup>10</sup> It is difficult to define any common character of these substances, save their power to injure the tissue with which they come into contact. A variety of bland substances have been tested with negative result; these include blood, blood serum (Flexner and Pearce);<sup>11</sup> agar-agar (Flexner), paraffin (Thiroloux,<sup>12</sup> Hess), emulsion of starch (Hess), etc.

<sup>5</sup> *Bull. internat. de l'acad. des sciences de Bohême*, 1898; and *Arch. Bohême*, 1890, iv, 139. Cited by Katz and Winkler, *Arch. f. Verdauungskr.*, 1896, iv, 289.

<sup>6</sup> *Bull. of the Johns Hopkins Hospital*, 1901, xii, 182.

<sup>7</sup> *Jour. of Exper. Med.*, 1906, viii, 167.

<sup>8</sup> *Berl. klin. Wchnschr.*, 1906, xliii, 1562.

<sup>9</sup> Contributions to the Science of Medicine. Dedicated to William H. Welch. *Johns Hopkins Hospital Reports*, 1900, ix, 743.

<sup>10</sup> *München. med. Wchnschr.*, 1903, I, 1905.

<sup>11</sup> *Univ. of Pennsylvania Med. Bull.*, 1901, xiv, 193.

<sup>12</sup> Thesis, Paris, 1892.

Especially significant is the fact that acute hemorrhagic pancreatitis produced by the methods which have been described, may be wholly unaccompanied by bacterial infection. Injection of cultures of various bacteria in small amount causes suppurative or chronic interstitial inflammation (Carnot<sup>13</sup> and others). Introduction of fluid cultures in considerable amount into the pancreatic duct may cause the hemorrhagic lesion which is perhaps referable, according to Hlava, to the acid products which they may contain.

Acute hemorrhagic pancreatitis in man has followed various injuries to the gland; fatal hemorrhagic or gangrenous pancreatitis it is well-known has followed immediately localized injury in the epigastrium, the kick of a horse, crushing of the body between the bumpers of two cars, passage of the wheel of a wagon over the body, etc., and in a few instances hemorrhagic pancreatitis with fat necrosis has occurred about the tract of a gunshot wound of the pancreas. Experimental studies have not fully explained such occurrences. A series of ligatures about the gland occluding both blood vessels and ducts causes a similar lesion, usually fatal within twenty-four hours (Katz and Winkler)<sup>14</sup> (Doberaurer)<sup>15</sup>. Injection of various substances which may act as emboli such as air, oil, paraffin (Bunge),<sup>16</sup> into the arteries of the gland cause necrosis of pancreatic parenchyma and of neighboring fat. Simple experimental crushing of the pancreas does not cause the lesion, but crushing associated with ligation of the blood vessels causes a hemorrhagic lesion accompanied by fat necrosis (Levin).<sup>17</sup>

Before attempting to show how these experiments explain the etiology of acute hemorrhagic pancreatitis in man, we wish to point out certain characters common both to the human and to the experimental lesions. Recognition of these peculiarities is essential to a clear understanding of the lesion, but they have not received the attention they deserve. Hemorrhage into the pancreas, believed to have much analogy to cerebral hemorrhage and caused perhaps by weakness or disease of the blood vessels of the gland, has often

<sup>13</sup> Thesis, Paris, 1898.

<sup>14</sup> *Arch. f. Verdauungskr.*, 1896, iv, 289.

<sup>15</sup> *Beitr. z. klin. Chir.*, 1906, xlviii, 456.

<sup>16</sup> *Arch. f. klin. Chir.*, 1903, lxxi, 726.

<sup>17</sup> *Jour. Med. Research*, 1907, xvi, 419.

been designated pancreatic apoplexy. There has been much discussion concerning the relation of hemorrhage and inflammation. Does inflammation occur as a consequence of hemorrhage, perhaps as the result of infection of hemorrhagic tissue, or is hemorrhage the result of severe inflammatory change? Study of the experimental lesion confirmed by the examination of human cases has rendered this discussion unnecessary, for it has shown that both hemorrhage and inflammation are secondary to necrosis of pancreatic parenchyma. The first effect of bile or other substance which causes the lesion, is death of pancreatic tissue with which the irritant comes in contact. Experiments of Flexner and Pearce have demonstrated the surprising rapidity with which this change occurs; one hour after injection of an irritant into the duct of the gland, hyaline necrosis has occurred and inflammatory products have found their way into the necrotic area. Such facts explain the sudden onset of the disease. One of us<sup>18</sup> has described the necrosis caused by injection of bile into the pancreatic duct of an animal, and has found that it affects not only the pancreatic cells, but the interstitial tissue of the gland together with the blood vessels which it contains. Necrosis of the walls of blood vessels explains the multiple hemorrhages which characterize the lesion. Inflammatory changes, moreover, occur only at the margin of the necrotic tissue. These observations show that the essential feature of the lesion is neither hemorrhage nor inflammation, but consists in death of large masses of pancreatic tissue. They emphasize the fact that acute hemorrhagic pancreatitis is not a bacterial infection like pancreatic abscess, but is primarily necrosis of the pancreas usually caused by contact of some irritant substance with the cells of the gland.

The fact that necrosis in mass is the essential feature of the lesion is well illustrated by Case II (p. 571) which is a typical instance of acute pancreatic necrosis accompanied by disseminated focal fat necrosis. This case is noteworthy because macroscopic hemorrhage is almost wholly wanting although the pancreatic parenchyma has undergone almost complete necrosis; a few black spots represent changed blood but there is no diffuse hemorrhagic infiltration of the necrotic tissue.

<sup>18</sup> *Bull. of the Johns Hopkins Hospital*, 1901, xii, 182.

The lesion usually described as acute hemorrhagic pancreatitis has not the characters of an inflammatory process. Widespread necrosis of pancreatic parenchyma is primary and such inflammatory changes as occur are found only at the margin of the necrotic tissue. A clearer understanding of the nature of the disease would doubtless result if the term hemorrhagic necrosis of the pancreas were used in place of acute hemorrhagic pancreatitis.

Similar lesions do not occur in other organs save, perhaps, in the stomach, where impaired vitality of the mucosa is followed by necrosis and subsequent ulceration, often with hemorrhage brought about by action of the gastric juice upon the injured tissue. The analogy is significant for the pancreas too contains an active proteolytic enzyme which unrestrained is capable of destroying tissue. The peculiar necrosis which affects the pancreatic parenchyma is doubtless referable to the trypsin which is contained in the pancreatic cells. Numerous studies have clearly demonstrated that the necrosis of fat which accompanies hemorrhagic necrosis of the pancreas is caused by a second enzyme, the fat-splitting enzyme of the pancreatic juice.

Recognition of the fact that necrosis of tissue is the essential feature of the acute hemorrhagic lesion, explains the relation of hemorrhagic to gangrenous pancreatitis. In individuals who die within from one to three or four days after onset of the symptoms, the gland is swollen and hemorrhagic, but when death occurs after a longer period there are changes in the hemorrhagic tissue so that it becomes black and gangrenous in appearance. It has been customary to describe as separate diseases hemorrhagic and gangrenous pancreatitis, whereas in both the underlying change is death of pancreatic parenchyma, and the two conditions represent two stages of the same lesion.

We have recently had the opportunity of studying three cases of acute hemorrhagic pancreatitis, which will be briefly cited because each one illustrates some important fact concerning the etiology or pathology of the disease.

CASE I.—M. K., female, aged 34 years, was admitted to the Presbyterian Hospital, in the service of Dr. Eliot, on November 8, 1908. She remembered no past illness. One brother died of acute pulmonary tuberculosis.

*Present Illness.*—In February, 1908, the patient was suddenly attacked with severe pain in the epigastrium. The pain was sharp and intermittent and was accompanied by vomiting which afforded some relief. The pain lasted about twenty-four hours and completely disappeared but returned after several days. There had been slight jaundice during the whole illness, but it was most marked during the three days before admission when the urine was dark colored. These symptoms continued for about three weeks, and disappeared. About October 1, 1908, she was again attacked with severe, sharp epigastric pain accompanied by vomiting. The pain was continuous and was increased only by deep respiration. There was frequent vomiting, but no jaundice; bowels were regular and feces were normal in appearance. About the middle of October the pain gradually diminished and almost disappeared. October 24, 1908, she had a severe chill followed by sweating.

On entrance to the Hospital the patient was well nourished but very pale; there was no jaundice. In the epigastrium to the right of the midline was a fluctuating, slightly tender mass, 8 cm. in diameter; the overlying skin had been reddened by poultices. The edge of the liver could be felt on either side of the midline and the mass seemed to be connected with this organ.

On November 9, 1908, Dr. Eliot opened the abdomen and evacuated a large abscess apparently connected with the liver. It contained a large quantity of thick, brownish pus, with very little odor. Agar-agar inoculated with this pus remained sterile. After operation the patient did not improve; she vomited frequently and was unable to retain any food. The wound discharged freely and at times masses of grayish, cheesy, necrotic material were found on the dressings. The finger could explore a cavity about 12 cm. in diameter with walls covered by soft necrotic material; to the left no wall could be felt. Microscopical examination of the mass discharged from the wound revealed only necrotic debris.

On November 30, 1908, a second operation was performed. A large cavity was found extending to the posterior parietes and for some distance both to the right and to the left of the midline. There was very little pus but the cavity was lined with necrotic material. The patient did not improve after the second operation and died December 1, 1908.

Temperature after admission to the Hospital ranged from 100 to 104° F., pulse from 100 to 160, and respiration from 20 to 60.

Urine contained a trace of albumin and a few casts; there was no bile nor sugar; there was no leucin nor tyrosin and the pancreatic reaction of Cammidge was negative.

The white blood corpuscles on entrance to the Hospital numbered 14,800 per c.cm. but diminished to 3,000 per c.cm. Progressive diminution of polynuclear leucocytes with increase of transitional forms, large mononuclear leucocytes and lymphocytes is exhibited by the counts on page 567.

*Autopsy.*—An autopsy was performed 3 hours after death. In the upper right hypochondrium from the wound of operation there is a deep sinus surrounded by firm adhesions.

In the fat of the omentum over the transverse colon there are numerous, small, white areas of fat necrosis. There are no such foci in the dependent part of the omentum. In the median line the coils of small intestines are adherent

Total leucocytes per c.cm .....	Nov. 8.	Nov. 16.	Nov. 18.	Nov. 27.	Nov. 28.	Nov. 30.
14,800	10,700	15,700	4,800	2,750	3,000	
Poly. leucocytes .....	78.5%	77 %	29%	26 %	41.5%	
Transitional .....	6	6	16	27.5	15	
Large mononuclears ..	2	2	21	4	16	
Lymphocytes .....	13	14.5	31	42	25.5	
Basophilic .....	0	0	0	0	0.5	
Eosinophilic .....	0	0	1	1	1.5	
Stimulation forms ....	0.5	0.5	2	0	0	

to the base of the mesocolon; after these adhesions are broken an abscess cavity, extending across the abdomen above the transverse mesocolon is exposed. At the base of the mesentery, especially where the loops of the small intestines are adherent to the transverse mesocolon are large plaques of fat necrosis. The gastro-colic omentum forms the anterior boundary of the abscess cavity. The hepatic flexure of the colon is firmly adherent to the liver.

From the opening in the abdominal wall the sinus with necrotic edges passed directly backwards to the duodenum. Over the surface of the duodenum the sinus with walls of black, soft tissue extends a short distance to the right; to the left it communicates with a very large cavity lying in front of the pancreas and representing in part the lesser peritoneal cavity. This cavity lies in great part below the pancreas and stomach which at the splenic end of the gland are tightly bound together. It extends to the left as far as the spleen and lateral abdominal wall. An extension from this cavity passes downward into the retro-peritoneal tissues to the left of the root of the mesentery, and in front of the kidney. Erosion in this region has extended into the abdominal wall and has reached to within about 1 cm. of the surface above the crest of the ilium.

Behind the body of the pancreas, extending upward through the retroperitoneal tissue is a second sinus which communicates with an immense cavity above the stomach and immediately below the diaphragm extending from the under surface of the liver to the spleen. The diaphragm to the left of the midline in contact with the abscess cavity is penetrated by an opening 1.5 cm. across. This opening communicates with a localized pleural abscess (7 by 8 cm.) about which the lung is firmly bound to the diaphragm; its wall is covered by shaggy fibrin and it contains foul smelling pus-like fluid. The pulmonary tissue in contact with this cavity is firmer than elsewhere.

In front of the head of the pancreas is the abscess cavity described above; the surface of the gland is here covered by soft, black tissue. This black gangrenous tissue covers the body of the pancreas half way to the splenic extremity whereas the remainder of the anterior surface of the body and tail is adherent to the stomach and shows neither necrosis nor erosion. The abscess cavity which has burrowed underneath the gland about its midpart dissects the greater part of the posterior surface of the splenic half from the underlying retroperitoneal tissue. The pancreas throughout is firm and on section the lobulation is less clearly defined than usual. On the surface and less frequently in the substance of the gland occur opaque, yellow spots of fat necrosis 2 or 3 mm. across. On section through the abscess wall into the head of the pancreas a narrow zone of fibrous tissue is found between the necrotic abscess wall and

the pancreatic parenchyma. The duct of the pancreas is slightly dilated and patulous.

On opening the duodenum the papilla of Vater is conspicuous and measures approximately 3 mm. in diameter; the circumference of the orifice is 12 mm. The common bile duct is somewhat dilated and 1 cm. above the orifice, measures 16 mm. in circumference. The duct of Wirsung joins the common bile duct 7 mm. from its duodenal orifice.

The gall bladder is small and its wall is thick. It contains eleven, small gall stones measuring from 6 to 9 mm. in diameter. One stone is partially enclosed by a saccule at the fundus. The cystic duct is dilated.



FIG. 1. Showing size of calculi found in gall bladder (Case I). Three stones of approximately same size as the smaller of those shown in the photograph were lost.

The liver weighs 2,275 gm. The surface in contact with the abscess cavity is discolored and covered by necrotic material. The substance is pale and the lobulation is indistinct.

Mucous membrane of the stomach is smooth and pale except for a few submucous hemorrhages. The duodenum is apparently normal. The jejunum is empty.

*Culture* from the peritoneum contains many varieties of bacteria, including streptococci and *B. coli* together with other unidentified bacteria. No culture grew from the liver.

*Microscopical examination* shows that the capsule of the pancreas is much thickened and its outer layers are necrotic. The inner layer consists of vascular granulation tissue in which are hemorrhages. Extending in from the capsule are greatly thickened strands and masses of similar tissue in which there are numerous round cells. In large areas parenchyma is almost wholly replaced and lobules are represented by a few acini. The islands of Langerhans are numerous and large; their capsules are somewhat thickened but there is no increase of connective tissue between the cells. The blood vessels are thickened and hyaline. The walls of the ducts are somewhat thickened; in places the lumen is dilated.

*Anatomical Diagnosis.*—Hemorrhagic necrosis of the pancreas in process of healing; disseminated fat necrosis; peri-pancreatic abscess with erosion of retroperitoneal tissue; subdiaphragmatic abscess; perforation of the diaphragm; localized diaphragmatic empyema; pneumothorax. Cholelithiasis; chronic cholangitis; fatty degeneration of the liver. Broncho-pneumonia. Fibro-myoma of the uterus.

Several features of the case are especially noteworthy: First, the gall-bladder contains eleven small stones of almost uniform size and the conformation of the common part of the biliary and pancreatic ducts is such that any one of these stones might block the duodenal orifice of the two ducts and divert bile into the pancreatic duct. Such entrance of bile into the pancreas would explain the gangrenous peripancreatitis found at autopsy. A second feature of considerable surgical interest is the tendency of the peripancreatic abscess, at first limited to the lesser peritoneal cavity, to erode the retroperitoneal tissues. The tendency of such abscesses to erode the tissue in front of the left kidney and to point in the lumbar region, is well illustrated by the present case. Incision in this region for counter-drainage has been at times practicable. The abscess cavity in the case just cited had moreover excavated a passage behind the pancreas, and had formed an immense subdiaphragmatic abscess between liver, stomach, diaphragm and spleen. Erosion of the diaphragm had caused complete perforation and had been followed by localized empyema. Such widespread solution of tissue is doubtless referable in part to the tryptic enzyme set free by the injured pancreas.

No growth of bacteria was obtained from the pus found at operation; cultures from the peritoneal cavity at autopsy contained various bacteria among which were *Bacillus coli* and streptococci. The leucocytes which during the first week after admission to the Hospital numbered approximately 15,000 per cu. mm. rapidly diminished in number and became subnormal just before death.

At autopsy there was no pancreatic necrosis but evidence of destruction of pancreatic parenchyma was furnished by bands of dense connective within the gland. When hemorrhagic necrosis produced experimentally in dogs is not fatal parenchyma which undergoes necrosis is absorbed and replaced by newly-formed fibrous

tissue. The same process has doubtless occurred in the case just described. Superficial necrosis of the pancreas with inflammatory changes has been followed by inflammation of the lesser peritoneal cavity with formation of the immense abscess, which has been described.

Several years ago one of us<sup>19</sup> showed that a *small* gallstone lodged at the duodenal orifice of the bile papilla might divert bile from the bile duct into the pancreatic duct. A large stone, on the contrary, would fill the common part of the two ducts and obstruct the outflow from both. The gall stone which causes the lesion usually escapes into the intestine and is lost. In the case of hemorrhagic pancreatitis which suggested the occurrence of the mechanism just described, the stone still occluded the common duodenal opening of the two ducts; the pancreatic duct was stained with bile. The earlier literature of the subject contains similar cases and almost identical instances have been recently described by Bunting,<sup>20</sup> and at a recent meeting of the New York Pathological Society, by Crowell. The gall stone which causes the pancreatic lesion usually escapes into the duodenum but in many recorded cases the gall-bladder has contained a large number of uniformly small stones, any one of which might cause the fatal diversion of bile into the pancreatic duct. In the case which we have just reported the gall-bladder contained such small calculi of almost uniform size (Fig. 1).

It has been claimed that gall stones are not more frequently found in association with acute hemorrhagic pancreatitis than in individuals of similar age dying from other diseases. Among eight cases of hemorrhagic necrosis (acute hemorrhagic pancreatitis), which I have had the opportunity of studying, gall stones have been present in five. Among one hundred and five recorded cases collected by Egdahl,<sup>21</sup> cholelithiasis was present in forty-two per cent. This proportion, which may be regarded as an established minimum, is doubtless small, for in many instances data which have been recorded do not exclude the presence of gall-stones. Their presence has not been noted or a single calculus causing the lesion has been

<sup>19</sup> *Loc. cit.*

<sup>20</sup> *Bull. of the Johns Hopkins Hosp.*, 1906, xvii, 265.

<sup>21</sup> *Idem*, 1907, xviii, 130.

lost. According to the statistics collected by Körte,<sup>22</sup> acute hemorrhagic and gangrenous pancreatitis occurs with few exceptions between the ages of twenty and sixty years. According to statistics of Mosher,<sup>23</sup> carefully compiled from German and American sources, the frequency of gall stones between the ages of twenty-one and sixty years is represented by 8.9 per cent. of all autopsies, less than one-fourth of the proportion found with the acute pancreatic lesion.

Whereas it is not improbable that half, or perhaps more than half, of all cases of hemorrhagic necrosis are caused by gall stones, all cases cannot be thus explained. In the following case which occurred in the service of Dr. Eliot at the Presbyterian Hospital, no gall stones were found at autopsy, and the conformation of the pancreatic ducts was such that a gall stone could not have produced the lesion.

CASE II. J. S., was admitted to the Presbyterian Hospital in the service of Dr. Eliot, on October 25, 1908. The patient was male, white, aged 55 years, a coach driver by occupation. One sister died with acute tuberculosis.

The patient has used alcohol in excess; in October, 1906, he had acute alcoholic gastritis and suffered with frequent vomiting and severe diarrhoea; there was hematemesis and considerable melena. The liver was palpable 4 cm. below the costal margin and tender; there was no jaundice. After this illness symptoms of chronic gastritis continued and there was frequent morning vomiting and eructations.

On October 22, 1908, there was sudden, severe epigastric pain followed by vomiting. The pain continued to be severe and extended a little outside the epigastric region. Vomiting was almost continuous and the vomitus had a very dark color but no foul odor. There was constipation. The patient had been dyspneic.

On entrance to the Hospital the patient was almost moribund; the face and hands were cyanotic; there was no jaundice. Temperature was 99.6° F., respiration, 40; pulse, 140. The abdomen was symmetrical and distended but not very tense; it moved little with respirations. There was tenderness and rigidity in the right upper quadrant and in less degree in the left upper quadrant. No mass was palpable. On percussion the right upper quadrant, the upper part of the right flank and the whole of the left flank were dull.

No urine was obtainable for examination.

White blood corpuscles October 25, numbered 9,900 per cu. cm. Differential count: polymorphonuclear leucocytes, 88 per cent.; transitionals, 5 per cent.;

<sup>22</sup> *Chirurgische Krankheiten des Pankreas. Deutsche Chirurgie, Stuttgart, 1898.*

<sup>23</sup> *Bull. of the Johns Hopkins Hospital, 1901, xii, 253.*

large mononuclear leucocytes, 1 per cent.; lymphocytes, 5 per cent. and eosinophiles 0.5 per cent.

Patient died six hours after entrance to the Hospital.

*Autopsy.*—An autopsy has been performed fifteen hour after death. The mesentery is studded with small grayish areas of fat necrosis. These areas are most numerous on the right side and many large foci are found in the perirenal fat.

The tail and body of the pancreas are soft, and have a deep, dark red color mottled with lighter, grayish areas. Here and there occur minute whitish spots of fat necrosis. Upon the surface of the body occur a few blackish spots extending into the substance of the gland; they represent the only evidence of hemorrhage. The greater part of the head of the gland shows the same changes and one or two black areas occur; the only part of the gland which has the appearance of normal tissue is situated on the posterior and lower part of the head in contact with the duct of Wirsung.

Liver weighs 3,425 gms. The surface is smooth; the cut surface has a uniform yellow color and the lobulation is distinct. The gall bladder, which measures  $11 \times 5\frac{1}{2}$  cm., is distended with rather thick, dark green bile, and contains no stones nor sand. The bile ducts are patent.

Stomach is large; its mucous membrane is in places injected and covered by blackish mucous. The duodenum is normal.

The kidneys show a moderate increase of interstitial tissue; there is general arteriosclerosis. At the apices of both lungs are a few encapsulated, partly caseous nodules. The other viscera are normal in appearance.

*Cultures* from spleen and liver contain *Bacillus coli*; aerobic and anaerobic cultures from the pancreas contain only *Bacillus coli*.

*Microscopical Examination of the Pancreas.*—A section from the head near the duct of Santorini shows complete necrosis and disintegration so that the architecture of the gland is completely lost. In a few small spots parenchyma is well preserved, acini being intact and nuclei well stained. Here interstitial tissue is distended as if by edema, red blood corpuscles are fairly numerous and polynuclear leucocytes occur in small number. At the margin of such areas acini still preserve their form but nuclei have disappeared. In some spots of preserved parenchyma there is slight increase of interstitial tissue. About the duct of Wirsung there is in places necrosis with loss of nuclei but except in a few areas the shape of the acini is recognizable, whereas in the greater part of the section tissue is intact and nuclei are stained. A section from the body shows almost complete disintegration similar to that about the duct of Santorini. An area of intact tissue shows considerable increase of connective tissue in great part about the lobules; this tissue contains numerous lymphoid cells and red blood corpuscles and a few polynuclear leucocytes. Tissue in the tail of the gland is in part necrotic and disintegrated, in part living. The preserved tissue is in places infiltrated with red blood corpuscles. Well preserved islands of Langerhans occur in fair abundance. Small veins contain thrombi.

*Anatomical Diagnosis.*—Hemorrhagic necrosis of the pancreas; chronic interstitial pancreatitis; fat necrosis; chronic pulmonary tuberculosis; chronic interstitial nephritis; fatty degeneration of the liver; arteriosclerosis.

In Case II death occurred only three days after onset of the symptoms which were such as usually accompany acute hemorrhagic necrosis of the pancreas. Pancreatic parenchyma has undergone almost complete necroses; there are trivial inflammatory changes at the margin of the necrotic tissue and in the neighboring abdominal fat are widely scattered foci of necrosis; the lesion has the characters found with so-called acute hemorrhagic pancreatitis yet hemorrhage is almost wholly wanting. The altered tissue is not visibly infiltrated with blood and minute black spots sparsely scattered upon the surface of the gland represent the only evidence of ecchymosis. Microscopic examination shows that there has been some escape of blood into the interstitial tissue of that parenchyma which is still intact.

The ducts of the pancreas exhibit an anomalous arrangement. The duct of Wirsung, which joins the common bile duct, is of small size and drains only a small part of the head of the gland; this area appears to be the least changed part of the organ. The duct of Santorini, which constantly enters the duodenum nearer the stomach than the duct of Wirsung, and is usually a small accessory duct (see Fig. 2), is in this instance the chief outlet of the gland, and, far larger than the duct of Wirsung, traverses the entire length of the pancreas (see Fig. 3). The orifice of this duct readily admits a probe about two millimeters in diameter. The gall-bladder and bile passages contain no calculi.

Since the duct of Wirsung is relatively small, whereas the main duct of the gland enters the duodenum about 1.5 cm. from the common bile duct, bile could enter only a small part of the pancreas. On the other hand, it is not improbable that an anomalous duct opening by a relatively wide orifice, might have been the portal of entry for material from the duodenum. The power of such material to cause acute hemorrhagic pancreatitis has been repeatedly shown by experiments. Hlava, who showed that acid gastric contents injected into the pancreatic duct causes the lesion, has suggested that antiperistaltic movements of the intestine might drive gastric or duodenal contents into the pancreatic ducts; there is no evidence that the lesion is produced in this way. The discovery of enterokinase in the intestinal juice has suggested that self-diges-

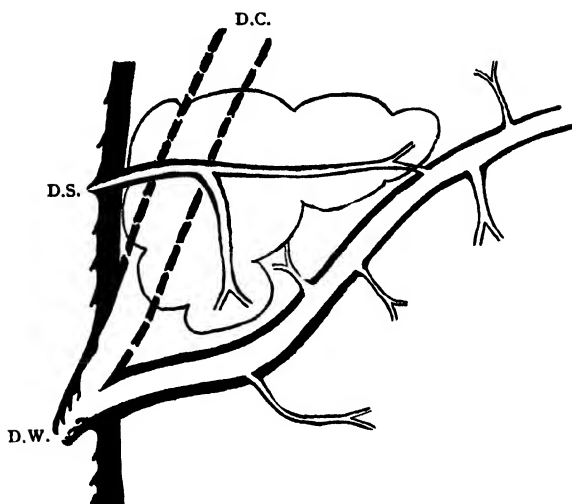


FIG. 2. Diagram showing the usual relative size of the duct of Wirsung (D.W.) and duct of Santorini (D.S.); the duct of Wirsung joins the common bile duct (D.C.).

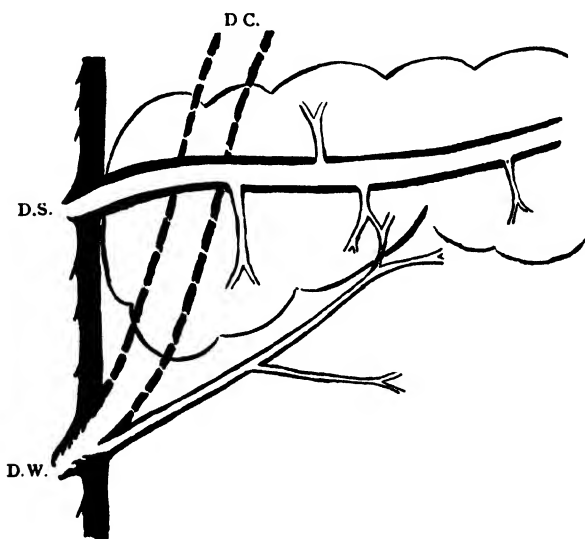


FIG. 3. Diagram showing the relative size of the two pancreatic ducts in Case II. The part of the gland drained by the duct of Santorini is indicated in both diagrams by curved lines.

tion of the pancreas might occur during life should enterokinase find its way into the pancreas. The part played by enterokinase is somewhat doubtful (Pólya, Williams and Busch),<sup>24</sup> but several experimenters (Pólya, Hess) have shown that small quantities of duodenal contents injected into the pancreatic duct will produce acute pancreatic necrosis with fat necrosis.

It is well known that increased pressure within the duodenum does not force duodenal contents into the bile duct or into the pancreatic duct after death. The delicate valves found within the diverticulum of Vater are believed to prevent regurgitation. In approximately one of ten individuals,<sup>25</sup> what is usually the accessory duct of the pancreas, namely, the duct of Santorini, is the chief outlet of the gland and is larger than the duct of Wirsung which joins the common bile duct (Fig. 3). The orifice of such an anomalous duct is perhaps unable to prevent regurgitation of intestinal contents when pressure within the duodenum is increased by vomiting. In the case which I have just described this anomaly has existed and perhaps explains the occurrence of the pancreatic lesion.

Cases recorded in the literature of the subject give some support to this view. Johnstone<sup>26</sup> has described two cases of acute pancreatitis in which the pancreatic duct has opened into the duodenum 1 or 2 cm. from the common bile duct, and although he has described these cases with the purpose of showing that gall stones have not been an etiological factor in the production of the lesion, he does not suggest that the anomalous condition may explain its occurrence. The description of this author makes it probable that the arrangement of the ducts in these two cases resembles that which I have just described. In an additional case described by the same author the distribution of the ducts is not described, whereas in a fourth case the nature of the lesion described is doubtful.

Further evidence that the duct of Santorini may be a portal of entry for irritant material from the duodenum is furnished by a case of Bassett.<sup>27</sup> The two ducts of the gland bore the usual relation to

<sup>24</sup> *Trans. of the Assn. of Am. Phys.*, 1907, xxii, 304.

<sup>25</sup> Opie, *Disease of the Pancreas*, Philadelphia, 1903, p. 30.

<sup>26</sup> *Colorado Med.*, 1907, iv, 93.

<sup>27</sup> *Trans. of the Chicago Path. Soc.*, 1907, vii, 83.

one another (Fig. 2); the duct of Santorini was much smaller than the duct of Wirsung and drained only a small part of the gland. This duct terminated at the margin of an anomalous duodenal diverticulum of which the mucosa was inflamed. Limited to the immediate neighborhood of this small duct was hemorrhagic pancreatitis with fat necrosis. The patency of the orifice of the duct of Santorini was not demonstrated. In the case which I have described almost the entire gland, of which the chief outlet was the duct of Santorini, was the seat of hemorrhagic inflammation, and the part about the small duct of Wirsung was apparently the least changed part of the organ, whereas in the case of Bassett, with a small duct of Santorini terminating in an anomalous diverticulum, the lesion was limited to the small area drained by this duct. (The domain of the duct of Santorini in the usual and in the anomalous condition is indicated in Figs. 2 and 3.) Although these cases do not afford conclusive proof that duodenal contents causes the lesion, they suggest this possibility with such force that a careful study of the topography of the ducts in cases of hemorrhagic necrosis of the pancreas is desirable.

The etiology in all instances of acute hemorrhagic pancreatitis is not the same. This fact is further emphasized by the occurrence of a small group of cases in which the lesion has so quickly followed abdominal injury in the epigastric region that its relation to traumatism cannot be doubted. A typical case is described by Selberg.<sup>28</sup> A man was kicked over the stomach by a horse and was unconscious for a time. There was pain and gradual distention in the epigastric region together with vomiting. Death occurred after twenty days. The omentum was studded with fat necrosis; the pancreas was infiltrated with blood and was gangrenous in appearance. In the following case localized hemorrhagic necrosis of the pancreas with localized fat necrosis followed a stab wound of the abdomen.

CASE III.—J. T., male, aged 20 years, was stabbed on the afternoon of November 4, 1908, in the upper left quadrant of the abdomen with a long stiletto. After about ten minutes he vomited, but there was no blood in the vomitus. Vomiting was followed by nausea. At the site of the wound there was considerable pain, which was increased by breathing.

<sup>28</sup> *Berl. klin. Wchnschr.*, 1901, xxxviii, 923.

On entrance to the Hospital a short time after the injury the temperature was 100.6°; pulse, 96; respirations, 16. The heart and lungs were found normal. Just above the costal margin in the left mammillary line there was a horizontal stab wound one inch long and parallel to the ribs; through the wound projected a mass of omentum, about the size of an egg. There was slight swelling about the wound but very little tenderness. There was no evidence of fluid in the abdomen.

Exploratory operation was performed November 4, 1908. The stab wound in the left costal space perforated the diaphragm, but the pleura was not injured. Upon the antero-lateral surface of the stomach was a perforation 1.5 cm. long through which a small amount of fluid had escaped. The posterior wall was apparently normal. The peritoneal cavity contained a large amount of fluid and clotted blood. The intestines and other viscera were apparently uninjured. The perforation in the anterior wall of the stomach was closed by sutures.

During the succeeding days the temperature varied between 101° and 106°, and the pulse was very rapid. There was frequent nausea and vomiting. There was distention of the abdomen, although the bowels moved freely and considerable flatus was expelled. Discharge from the wound was profuse. The patient gradually became weaker and died November 9.

Streptococci in pure culture were found in the peritoneal exudate. A blood culture remained sterile.

White blood corpuscles numbered from 3,000 to 7,000 per c.mm.

The urine contained a faint trace of albumin and a few casts.

*Autopsy* was performed by Dr. Schutz, coroner's physician. Throughout the peritoneal cavity is fibrino-purulent exudate. The wound of the antero-lateral wall of the stomach has remained closed and is in process of healing. There is no perforation of the posterior wall of the stomach, but opposite to the wound in the anterior wall there is upon the mucosa of the posterior wall a circular spot of edema and injection 2.5 cm. in diameter; a minute central area of necrosis about the size of a pin's head, evidently represents the spot in which the point of the knife had struck. Upon the surface of the pancreas immediately behind this wound is a localized hemorrhagic area about 3.5 cm. in diameter and in the fat of this area are conspicuous foci of fat necrosis. Otherwise the pancreas appeared normal.

Microscopic examination of the hemorrhagic area in the pancreas shows hyaline necrosis of the pancreatic parenchyma similar to that frequently observed with so-called acute hemorrhagic pancreatitis; the interstitial tissue is infiltrated with blood and in places contains polynuclear leucocytes. The veins within the hemorrhagic zone are widely dilated and contain thrombi.

It is noteworthy that simple injury to the pancreas of animals fails to produce necrosis, whereas injury associated with occlusion of blood vessels is followed by changes which resemble those of hemorrhagic necrosis (see p. 563). Trivial injury to the pancreas is usually followed by rapid healing but in Case III injury was associated with pancreatic necrosis, hemorrhage, slight inflammation

and focal fat necrosis. It is not improbable that the simultaneous occurrence of localized venous thrombosis and pancreatic injury, both due to a stab wound, explains the occurrence of hemorrhagic necrosis of pancreatic parenchyma.

The lesion usually designated acute hemorrhagic pancreatitis is primarily necrosis of the pancreatic parenchyma and may be caused by various, usually chemical, occasionally mechanical, injuries to the gland. The name hemorrhagic necrosis is preferable to acute hemorrhagic pancreatitis because the lesion is essentially widespread necrosis of the pancreatic parenchyma and the inflammatory changes which occur are secondary to necrosis or subsequent to bacterial infection.

The pancreas is not more susceptible to spontaneous hemorrhage than other organs; so-called pancreatic apoplexy is the result of acute pancreatic necrosis. In some instances pancreatic necrosis (Case II) may cause little if any hemorrhage. So-called gangrenous pancreatitis is a late stage of hemorrhagic necrosis.

Hemorrhagic necrosis of the pancreas is not primarily the result of bacterial infection, but in some instances subsequent infection of gangrenous tissue may cause suppuration.

The most frequent cause of hemorrhagic necrosis of the pancreas in man is penetration of irritant material into the ducts of the pancreas. Bile diverted by a gall stone lodged at the duodenal orifice of the common bile duct has produced the lesion in a large proportion of cases; duodenal contents entering the duct may have the same result. In a small proportion of cases pancreatic necrosis follows injury to the gland and is perhaps in part referable to simultaneous thrombosis of blood vessels.

Certain individuals are rendered susceptible to hemorrhagic necrosis of the pancreas by anatomical peculiarities or anomalies of their pancreatic ducts. In some individuals the passage of a gall stone may divert bile into the pancreas; in others perhaps the structure of the ducts may be such that duodenal contents can find its way into the pancreatic ducts, and thus cause the disease.





## OBSERVATIONS ON URICOLYSIS, WITH PARTICULAR REFERENCE TO THE PATHOGENESIS OF "URIC ACID INFARCTS" IN THE KIDNEY OF THE NEW-BORN.<sup>1</sup>

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The fact that deposits of urates are found in the collecting tubules of the kidneys of about one-half of all infants dying during the first two weeks of life, indicates that, in all probability, similar deposits occur frequently in normal infants. Consequently, it must be assumed that such deposits are not often a source of any harm, which assumption accounts for the relatively slight consideration given to these so-called "uric acid infarcts of the new-born." Nevertheless, it seems improbable that deposition in the renal tubules of masses of crystals of ammonium urate can always fail to cause injury, for in the first place these substances are not altogether non-toxic, if we accept the results of the studies of Freudweiler,<sup>2</sup> His,<sup>3</sup> and others who have shown that urates cause local necrosis and act as weak tissue poisons. Furthermore, the very slight solubility of uric acid and the urates insures their remaining in the tubules for some time after they are once deposited; Kaufmann indeed mentioning their persistence for years, finding them in one case in a boy seven years old. Certainly they may serve as the starting point of urate calculi, which occur not infrequently in young children, and it seems not improbable that injury to the renal tissue by these deposits may serve as a starting point for infection. Suppurative pyelitis in infants and young children has been frequently observed, due

<sup>1</sup> This work has been aided by a grant from the Rockefeller Institute for Medical Research.

<sup>2</sup> Freudweiler: *Deutsch. Arch. f. klin. Med.*, lxiii, p. 266, 1899.

<sup>3</sup> His: *Ibid.*, lxvii, p. 81, 1900.

to infection with the colon bacillus as a rule, and no more probable explanation for the localization of the bacilli in the pelvis of the kidney presents itself than that the urate deposits have furnished a *locus minoris resistentiæ*. We have also observed a case which suggests that hemorrhagic infarction of the kidney of the new-born may, at least in some instances, be the result of infection of the pyramids by *B. coli*, presumably localized there for the reasons cited.<sup>1</sup>

Whether actually important as a possible cause of serious harm, as suggested above, or simply a harmless, transitory deposit of urinary constituents in the renal tubules, this process has a considerable interest through its bearing upon the general problems of the physiology and pathology of uric acid metabolism. Many puzzling features offer themselves, the solution of which may throw light on other questions concerning uric acid and its fate. Why are these deposits so common immediately after birth and so uncommon at other times? Why are they observed only in new-born human offspring?<sup>2</sup>

Virchow considered that at the time of birth there resulted a transformation in the entire metabolism on account of the taking in of nourishment, the introduction of direct respiration, and the first exercising of the thermo-regulatory functions, and this manifested itself, among other ways, in an increased elimination of uric acid. Vierordt believed that the feebleness of the oxidation processes during the first days of life was responsible for the excessive output of uric acid, although the direct evidence as to actual oxidizing activity of the new-born human being is not at all clearly in favor of this view. Ebstein thought that in addition to the increased excretion of uric acid another factor was necessary in order to bring about the precipitation of the uric acid in the tubules, and this factor he found in the degenerated renal epithelium, which is injured by the excessive uric acid. Of similar purport is the contention of Flensburg<sup>3</sup> that the tubules

<sup>1</sup> Wells: *Trans. Chicago Path. Soc.*, vii, p. 242, 1909.

<sup>2</sup> The only exception that we have found is the statement by Spiegelberg (*Arch. f. exp. Path. u. Pharm.*, xli, p. 428, 1898) that Pohl observed typical "uric acid infarcts" in the kidney of a monkey which was but a few weeks old.

<sup>3</sup> Inaug. Dissert., Stockholm, 1893, abstracted in Maly's *Jahresber. f. Thierchem.*, xxiii, p. 581, 1893.

of the new-born secrete a hyaline substance which acts as a matrix for urate deposition. Whatever this hyaline matrix is, it would seem to be a colloid forming reversible gels or precipitates, for, as pointed out by Schade,<sup>1</sup> if the colloidal matrix of a urinary precipitate is reversible the precipitate is readily redissolved and does not tend to form calculi, as is the case with the uratic deposits of the new-born; when the colloidal matrix is one which forms non-reversible precipitates, such as fibrin, the deposit is not readily dissolved and forms true concretions.

There is no doubt that the urine of the newly born infant does contain much more uric acid proportionately than the urine of the adult, and this may be ascribed either to excessive formation of uric acid or to a defective destruction. Sjöqvist found that in the new-born the ratio of urea nitrogen to uric acid nitrogen is 74.9 to 7.9, whereas in the adult the ratio is about 85 to 2, and other observers have noticed the relatively large quantity of uric acid in the urine of infants. To account for this increase in uric acid a number of hypotheses have been advanced, most prominent being the view that there is a large and rapid destruction of leucocytes about the time of birth, yielding from their nuclear material the antecedents of the uric acid. We may also imagine that the transformation of the nucleated red corpuscles of the fetus into the non-nucleated corpuscles of the infant gives rise to excessive quantities of free purines. On the other hand a similar result would be obtained if the power of the body to destroy uric acid were decreased, whether by generally defective oxidation as suggested by Vierordt, or from some other more specific cause. In any case the urinary uric acid of the infant must be largely of endogenous origin, for milk is extremely poor in purines.<sup>2</sup>

The fact that the urates are deposited in the collecting tubules may be looked upon as a demonstration of absorption of water in these tubules, since the urates, being relatively insoluble, are precipitated when the dilute urine of the convoluted tubules is concentrated by absorption in the collecting tubules.

<sup>1</sup> Schade: *Münch. med. Wochenschr.*, lvi, p. 3, 1909.

<sup>2</sup> See Orgler: *Ueber Harnsäureausscheidung im Säuglingsalter*, *Jahrb. f. Kinderheilk.*, lxxvii, p. 282, 1908.

Recent studies of purine metabolism have put the matter of uric acid destruction upon a more definite basis than it formerly occupied, and we now know that this destruction is accomplished by definite uricolytic enzymes which have a widespread but irregular occurrence, both as regards various animal tissues and various animal species. For example the bovine kidney is actively uricolytic, while the bovine spleen is devoid of this effect. Similarly we have found, in connection with another study to be published separately, that the spleen of the dog does not destroy uric acid while the liver is very energetically uricolytic. Also there is a difference in the distribution of uricolytic enzymes in the animal kingdom. In an invertebrate, a mollusk, studied by Mendel and Wells<sup>1</sup> it was found that not only was there no uricolytic power, but also that the enzyme, *xanthoöxidase*, which forms uric acid from the oxy-purines, is lacking. The liver of birds will not destroy uric acid perfused through it,<sup>2</sup> as is to be expected from the fact that nitrogenous elimination in the birds is chiefly in the form of uric acid, and in the tissues of the turtle, as an example of a reptile, we have also found complete absence of uricolytic power. On the other hand most mammalian organisms seem to possess uricolytic enzymes, although this is apparently not universal, a fact which will be considered later.

Wiechowski and Wiener<sup>3</sup> give the following as the distribution of uricolytic enzymes in different animal species, according to the collected results of various authors: In bovines, in the kidney, muscles, liver, perhaps in the bone-marrow, but not in the spleen, lung or intestines; in dogs, in the liver but not in the kidneys; in the liver of pigs; in the kidneys and many other organs of the horse; in the liver but not in the kidneys of rabbits. To this list we may add from our own observations, that the liver of the guinea-pig is actively uricolytic, that the liver and the other viscera of the turtle seem to be entirely inactive, and that the spleen, bone-marrow and probably the leucocytes of the dog are not uricolytic.

<sup>1</sup> Mendel and Wells: *Amer. Journ. of Physiol.*, xxiv, p. 170, 1909.

<sup>2</sup> Friedman and Mandel: *Arch. f. exp. Path. u. Pharm.*, Supplement-band, 1908, p. 199.

<sup>3</sup> *Beitr. z. chem. Physiol. u. Pathol.*, ix, p. 247, 1907.

The activity of this enzyme is very considerable; beef kidney, for example, will totally destroy the uric acid added to it, if not too great in amount, in from four to seven hours.<sup>1</sup> However, in this respect it stands behind the remarkable rapidity of some of the other enzymes concerned in purine metabolism, for Schittenhelm<sup>2</sup> has observed quantitative conversion of guanine into uric acid by bovine spleen in one to two hours.

Mendel and Mitchell<sup>3</sup> have studied the development of the enzymes of purine metabolism in the developing pig embryo, with the following results: Nuclease appears at an early period, the power to free purines from the tissues during autolysis being present in 50 mm. embryos, the smallest studied. Adenase is present at the same early stage, but xantho-oxidase does not manifest itself in the livers of fetal pigs of 200 mm. length, although present in the liver of sucking pigs. Likewise uricolytic enzymes were not present in 200 mm. embryos, and only slight uricolytic power was demonstrable in the liver of sucking pigs, about two months old, although the liver of adult pigs is actively uricolytic.

These results obtained in a typically omnivorous animal, the pig, if transcribed to another omnivorous animal, man, would seem to offer a ready explanation of the high uric acid content of the infant's urine and the resulting uric acid infarcts in the kidneys. They suggest that during fetal life any necessary uricolysis is performed for the fetus by the mother, either in the placenta or after absorption of the uric acid of the fetal circulation into the maternal blood. After birth the uricolytic power, which does not appear until about this time, is more or less imperfectly developed for a few days or weeks, during which time uric acid that should be destroyed is excreted in the urine. In support of this theorization are the results obtained by Spiegelberg<sup>4</sup> with dogs. He saturated the tissues of dogs, both young and adult, with uric acid by subcutaneous injection, and found that the power of young dogs to destroy uric acid was much less

<sup>1</sup> Künzel and Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, v, p. 389, 1908.

<sup>2</sup> Schittenhelm: *Zeitschr. f. physiol. Chem.*, lvi, p. 21, 1908.

<sup>3</sup> Mendel and Mitchell: *Amer. Journ. of Physiol.*, xx, p. 97, 1907.

<sup>4</sup> Spiegelberg: *Arch. f. exp. Path. u. Pharm.*, xli, p. 428, 1898.

than that of old dogs. For example, when 0.1 gram of uric acid per kilo of body weight was injected into puppies, 53 per cent could be recovered in the urine, while with adult dogs under the same condition but 5.6 per cent was excreted. Also the urine of puppies becomes turbid with urates, and uratic infarcts of typical appearance are produced in the kidneys when but 0.05 to 0.1 gram uric acid per kilo is injected, whereas much larger quantities of uric acid cause no similar effects in adult dogs. Therefore it would seem that in young dogs the power to destroy uric acid is much less than in adult dogs, the possibility that the turbidity of the urine and the renal infarcts are due to poor solvent action of the urine in the young animal having been excluded.

With the object of testing this hypothesis, namely, that the high uric acid excretion and the occurrence of uric acid infarcts in young infants is due to the failure of development of uricolytic enzymes until after birth, as in the pig, a series of experiments was undertaken. The only similar experiments recorded in the literature are those of Schittenhelm and Schmidt,<sup>1</sup> which were as follows:

1. To the ground tissue of the kidneys (25 grams) of a full-term child, 0.1 gram uric acid was added, and autolysis was permitted for 4 days with frequent shaking for the purpose of aeration. At the end of this time no uric acid could be recovered.
2. 0.1 gram uric acid added to the extract of the kidneys (31 grams) of a full-term infant, under the same conditions. 0.078 gram uric acid recovered.
3. 140 grams of liver extract of an infant (age not stated) autolyzed with 0.3 gram of uric acid, a current of air being conducted through the mixture for seven days. No uric acid recovered.
4. 82 grams ground muscle from a seven-month's fetus and 0.2 gram uric acid autolyzed five days, with frequent shaking. No uric acid recovered.
5. 70 grams ground muscle from a full-term infant autolyzed with 0.2 gram uric acid for 14 days, frequently shaken. No uric acid recovered.
6. 58 grams of lung extract from a full-term fetus were autolyzed with 0.15 gram uric acid for 14 days, frequently shaken. 0.05 gram uric acid was recovered.

They further state that "We can add to these results that we obtained a questionable result with intestine emulsion. Unfor-

<sup>1</sup> *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 424, 1907.

tunately, material was lacking to repeat the experiments with lung and intestine. In any case these two organs, even if they possess any uricolytic power, stand much below the kidney, liver and muscle in activity. These three organs possess a very intensively acting uricolytic enzyme, agreeing with the organs of mammalian animals in which it is also found that the liver, kidney and muscle accomplish the destruction of uric acid."

These experiments would seem at once to give a negative answer to the possibility of explaining the conditions of uricolysis in the human infant on the basis of the results obtained by Mendel and Mitchell with pig embryos, for they indicate that the organs of the human fetus at term are able to destroy uric acid with great activity. However, there still remained the possibility that this uricolytic power develops late in fetal life, and that it is not always present at birth, and also, because of the unexpected result of these experiments of Schittenhelm and Schmidt, it seemed desirable to repeat them, and also to study embryos of different ages to ascertain the time at which the uricolytic power appears. It was not until we had already begun upon the work that we became acquainted with a later paper from Schittenhelm<sup>1</sup> which made the positive results of Schittenhelm and Schmidt seem even more surprising, and repetition more necessary. In this later paper, dealing with purine metabolism in adult man, it is reported that human liver, although actively oxidizing purines to uric acid, has little if any uricolytic power. They were not able to obtain satisfactory results from human kidneys. Human intestine was found to transform guanine into xanthine. They also state that, in a communication at that time unpublished, Wiechowski had likewise reported that he was unable to demonstrate any considerable evidence of uricolysis in human tissues, that he could find no allantoin in the urine after subcutaneous injection of uric acid, and that as 60 to 80 per cent of the uric acid reappeared in the urine he concluded that uric acid is not attacked by human tissues.<sup>2</sup>

<sup>1</sup> Künzel and Schittenhelm: *Zentralb. f. Physiol. u. Pathol. d. Stoffwechsels*, iii, p. 721, 1908.

<sup>2</sup> This article has since been published, *Arch. f. exp. Path. u. Pharm.*, ix, p. 185, 1909.

It would seem strange indeed that the new-born infant should have a well-developed power to destroy uric acid, as Schittenhelm and Schmidt maintain, while the adult has lost this capacity, yet Schittenhelm does not seem to consider this discrepancy. In one of his most recent articles<sup>1</sup> he refers to the previous observations on uric acid destruction by the tissues of the new-born, and considers them as conclusively proving that such human tissues are actively uricolytic.

The results of our own experiments, which are recounted in detail below, seem to be quite conclusive in showing that adult human tissues have no distinct power to destroy uric acid *in vitro*, thus agreeing with Künzel and Schittenhelm and with Wiechowski; furthermore, with fetal tissues we obtain results entirely the opposite from the paradoxical findings of Schittenhelm and Schmidt, for we find the human fetus at all stages of its development is devoid of any appreciable uricolytic power in its chief tissues and organs. Our method of experimentation was as follows:

The organ or tissue to be examined was ground fine in a hashing machine, placed in three volumes of toluol water and let autolyze at room temperature over night, then strained through cheese cloth. To the emulsion was then added the uric acid, which had been dissolved in boiling water by adding, drop by drop, just enough dilute sodium hydroxide to complete solution, and then quickly cooled. The mixture was then allowed to autolyze in a thermostat at about 37° in the presence of abundant toluol, a current of air being drawn through. After 24 or 48 hours, usually, the autolysis was stopped by boiling the mixture, coagulated with a minimum of acetic acid, filtered, and the residue well washed by being boiled with fresh water, alkalized faintly with sodium carbonate to assure solution of all residual uric acid, reacidified and refiltered and washed. In the filtrate the uric acid is precipitated by the copper sulphate and sodium bisulphite method of Krüger and Solomon, filtered, washed, decomposed with hydrogen sulphide, filtered, concentrated to 50 cc. in the presence of hydrochloric acid, and weighed directly after filtering off in a weighed Gooch crucible. When the precipitate gave any indication of any impurity it was repurified by

<sup>1</sup> Brugsch and Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, v, p. 406, 1908.

precipitating from the solution in concentrated sulphuric acid. After making the usual correction for solubility, control experiments with boiled tissue extracts showed that from 85 to 95 per cent of the added uric acid can be recovered by this method, the losses probably being partly due to retention in the precipitates, partly to losses in the copper sulphate precipitation and decomposition and partly, perhaps, to the destructive action of the alkali used in dissolving the uric acid. These slight losses are, however, of little significance, for we have always found that when a tissue has any uricolytic power at all this is always capable of destroying all the uric acid present under the conditions of these experiments. Therefore the isolation and weighing of the uric acid is really little more than a control procedure, for when tissues which are uricolytic are used there is no precipitate whatever when the copper sulphate is added to the filtrate, in marked contrast to the voluminous precipitate produced in the filtrate when there has been no uricolysis.

#### *A. Uricolysis by Adult Human Tissues.*

I. Tissues from the body of a man, aged 40, who died suddenly of heart disease. Twelve hours after death the organs, showing no visible pathological changes, were treated as above, 100 grams of tissue being used with each, the spleen, kidney and liver, and 75 grams of muscle tissue. Autolysis continued 48 hours with a current of air passing through the autolyzing mixture. From all the uric acid was recovered in large amounts, as follows:

Tissue.	Uric Acid Added.	Uric Acid Recovered.	Per Cent Recovered.
	Grams.	Grams.	
Spleen.....	0.1794	0.160	89
Kidney .....	0.1640	0.135	82
Liver.....	0.1700	0.185	109*
Muscle.....	0.1740	0.1815	76

\* Presumably this slight gain in uric acid depends upon the power of human liver to form uric acid from xanthine present in autolyzing extracts.

II. Tissues from body of a healthy man, aged 22, dying from a stab wound, and autopsied almost immediately after death. 100 grams of tissue used for each experiment, including one with liver extract heated to 100° for fifteen minutes. Autolysis with air current for 48 hours. Results:

Tissue.	Uric Acid Added.	Uric Acid Recovered.	Per Cent Recovered.
	Grams.	Grams.	
Kidney .....	0.1258	0.1102	87
Spleen.....	0.1186	0.0958	81*
Liver (fresh).....	0.1953	0.1833	93
Liver (boiled).....	0.1019	0.0988	97

\* Result of this analysis uncertain, as there was some slight loss. ,

#### B. Experiments with Fetal Tissues.

III. Female fetus, weight 727 grams, length 34 cm., therefore presumably about the sixth or seventh month. Liver weighed 34 grams, and was allowed to autolyze with 0.1896 gram uric acid for 48 hours with constant air current. Recovered 0.1821 gram uric acid, or 96 per cent; therefore no uricolysis.

IV. A fetus weighing 105 grams, length 16 cm., therefore probably about the fourth month. This was permitted to autolyze 24 hours with uric acid (with air current) and for control 50 grams of dog liver and 85 grams of guinea-pig liver (5 livers) treated in the same way with the following results:

Tissue.	Uric Acid Added.	Uric Acid Recovered.	Per Cent Recovered.
	Grams.	Grams.	
Fetus.....	0.1136	0.0834	73.4
Dog liver.....	0.1060	0.0	0
Guinea-pig liver .....	0.1319	0.0	0

V. Fetus, length 23 cm., weight 306 grams, presumably about the fifth month. Entire fetus ground up and extracted in the

usual way. Extract divided into two equal parts. Autolysis for 48 hours in air current at 37°, with following results:

(1). 150 grams tissue—added 0.1445 gram uric acid. Recovered 0.1393 gram uric acid or 96 per cent.

(2). 150 grams tissue—added 0.1405 gram xanthine. Recovered 0.1407 gram xanthine.

The recovery of as much xanthine as was added, without making any correction for loss by solubility or in manipulation, shows the absence of xantho-oxidase, and also suggests that some xanthine may have been formed from the tissue purines under the conditions of the experiment. Other experiments, to be reported later, show that from a very early stage the human fetus has the power of forming xanthine from guanine.

VI. Full term fetus, well developed, weight 3400 grams. Apparently died from asphyxiation because of podalic delivery without attendance. Made extracts of 150 grams of mixed viscera (including spleen, lungs, thymus, adrenals, pancreas and gastro-intestinal tract), 143 grams of muscle and 24 grams of kidneys, and let the three extracts autolyze with uric acid 48 hours in an air current. On account of a back flow of the material from one flask into another of the series the results with each tissue cannot be distinguished, but as to the three lots 0.372 gram of uric acid had been added and the total amount recovered 0.347 gram or 93 per cent; it is evident that there was no destruction of uric acid by any of these tissues.

VII. Full term fetus, living but three hours on account of congenital heart defect. Experiment performed in the usual manner.

Tissue.	Material Added.	Material Recovered.	Per Cent Recovered.
Grams.	Grams.	Grams.	
1. Liver (100)...	0.1514 uric acid	0.2002 uric acid	132
2. Liver (100)...	0.0958 xanthine	0.0856 uric acid	
3. Viscera (95)...	0.1514 uric acid	0.1706 uric acid	112
4. Viscera (95)...	0.0958 xanthine	0.0463 uric acid	
		0.0687 xanthine	
5. Kidney (22)...	0.1514 uric acid	0.1488 uric acid	98

This experiment demonstrates at one and the same time the presence of xantho-oxidase and the absence of uricolytic enzymes in the tissues of the full term fetus. The xantho-oxidase of the liver has converted the xanthine which was added into uric acid, which is then not destroyed, and it has also converted the purines present in the autolyzing liver tissue into uric acid. With the mixed viscera the conversion of xanthine into uric acid has not been so complete, possibly because of the fact, pointed out by Schittenhelm, that the enzymes of one organ may inhibit or destroy the oxidases of another organ. The time of development of xantho-oxidase in the human fetus will be more fully discussed in a later paper.

### C. Uricolytic Action of Placenta.

As Wiechowski<sup>1</sup> in particular has pointed out, the animals which destroy uric acid excrete allantoin in their urine as a product of this uricolysis. The older literature states that allantoin may be found in the urine of pregnant women (Gusserow) and of infants during the first few days after birth, but apparently it is not ordinarily present in the urine of adults. As we have found that the tissues of neither infants nor adults destroy uric acid, and hence presumably produce no allantoin, the possibility presented itself that the placenta might have uricolytic power, an assumption which fits in perfectly with the classical finding of allantoin in the amniotic fluid (cow) as well as with its reputed presence in the urine of pregnant women. Our experiments, however, showed absolutely no evidence of uricolytic power in the human placenta. Two fresh human placentas were obtained, ground up, and 250 grams used for each of the following experiments:

Tissue.	Uric Acid Added.	Uric Acid Recovered.	Per Cent Recovered.
	Grams.	Grams.	
1. Placenta (boiled).....	0.1427	0.1372	96
2. Placenta (fresh).....	0.1513	0.1467	97
3. Placenta (fresh).....	0.1486	0.1399	94

<sup>1</sup> *Arch. f. exp. Path. u. Pharm.*, lx, p. 185, 1909.

1 and 2 represented the extract from one placenta; 3 was from a different placenta.

The failure to demonstrate the presence of uricolytic power in human placenta as well as in other human tissues, causes some question to arise as to the validity of the claims that allantoin may be present in the urine of pregnant women. Wiechowski, in the article cited above, discusses these claims. He could find no authority whatever for the current statements that human amniotic fluid and the urine of infants during the first eight days of life contain allantoin, and he finds reason to doubt the methods by which allantoin is supposed to have been demonstrated in human urine. The negative result of studies of uricolysis by human tissues, including placenta, affords still further ground for skepticism regarding the occurrence of allantoin in the urine during pregnancy, and makes necessary a re-investigation of this question by more modern methods.

#### *D. Influence of Serum upon Uricolysis.*

It having been demonstrated in many ways that fresh blood serum exercises an inhibiting effect upon the ordinary processes of autolysis, and as uricolysis may be considered as an analogous process to the extent that it is a destruction of organic compounds by intracellular enzymes, it seemed possible that the negative results obtained in these experiments might perhaps be dependent in some measure upon an anti-uricolytic action of the serum present in the tissue extracts. An experiment was performed to decide this question, with negative results.

Fresh dog liver, weighing 250 grams, was ground up and let stand over night in toluol water at room temperature. Strained through cloth, made up to 400 cc., and divided into four 100 cc. portions. These were then let autolyze for 17 hours at 37° with constant air current, with the following additions:

1. Added 200 cc. water and 0.0966 gram uric acid. Result—No uric acid could be recovered.

2. Added 200 cc. water, then heated to 100° for 15 minutes, cooled, and added 0.1034 gram uric acid. Result—Recovered 0.0833 gram uric acid, or 86 per cent.

3. Added 200 cc. dog serum, freshly obtained, and 0.1175 gram uric acid. Result—No uric acid could be recovered.

4. Added 200 cc. of serum that had been heated at 75° for 20 minutes, and 0.0848 gram uric acid. Result—No uric acid could be recovered.

This experiment shows the power of dog liver to quantitatively destroy uric acid in a much shorter time than our experiments were continued with human tissues, and indicates that dog serum does not check the uricolytic power under the conditions of the experiment. It is possible that a much larger quantity of serum might inhibit uricolysis, or that the rate of uricolysis is slowed during the few hours needed for its completion, but it was not considered advisable to follow up this matter at this time, since the results of this experiment indicate that the quantity of serum present in the tissues with which we were working can not account for the total lack of uricolytic activity observed. The fact that in the living dog when the uric acid is parenterally injected it is rapidly destroyed by tissues that are bathed in constantly changing serum, makes it improbable, *a priori*, that dog serum can inhibit uricolysis by dog's tissues to any great extent. We have not attempted to learn whether human tissues washed free from serum have any more power to produce uricolysis than tissues in the presence of such small amounts of serum as are admixed with the organ after its removal from the body—that is, to finally establish that the failure of uricolysis by human tissues is due to lack of uricolytic enzymes, as seems probable, rather than to any highly developed anti-uricolytic property present in human serum and absent in dog serum.

#### *E. Uricolytic Action of Leucocytes of Dog.*

Earlier investigators of gout and uric acid have sought for a uricolytic action on the part of the blood, with negative results. Thinking that possibly the leucocytes of the dog, in which animal we have very active uricolytic enzymes in the liver, might possess uricolytic power, and having already demonstrated the absence of uricolysis in the spleen of the dog, an experiment with leucocytes and bone-marrow was performed.

A large adult dog received injections of an emulsion of 15 grams aleuronat, 5 cc. turpentine, and 150 cc. water, one-third into the pleura and two-thirds into the peritoneum. Killed

after 18 hours, and 500 cc. turbid, blood-stained exudate removed from the peritoneum, but none was obtained from the pleura. Left the fluid standing over night on ice, drew off the supernatant serum and obtained a sediment of 60 cc. of leucocytes mixed with red corpuscles and a little serum. The ribs of several dogs were removed, cleaned free from adherent tissue, ground up, extracted over night in toluol water, and the resulting emulsions of marrow tissue strained through cheese cloth. The leucocytes and the marrow extract were allowed to act upon uric acid in the usual way for 48 hours, and found not to destroy it. There being somewhat more than the usual loss of uric acid in the marrow extract this experiment was repeated twice with similar results, although in the last experiment the result was not different from that frequently obtained with other non-uricolytic tissues.

Tissues	Uric Acid Added.	Uric Acid Recovered.	Per Cent Recovered.
	Grams.	Grams.	
1. Leucocytes.....	0.0795	0.0716	90
2. Marrow.....	0.1590	0.1129	71
3. Marrow.....	0.1580	0.1146	73
4 Marrow.....	0.1543	0.1317	85

#### CONCLUSIONS.

From the foregoing experiments, together with recent observations by other investigators, it seems well established that the tissues of the adult human being possess no uricolytic enzymes, or at least none capable of producing appreciable uricolysis under conditions that give total destruction of uric acid by tissues from many other mammals. If further studies including all the tissues of the human body show a total absence of uricolytic power, which seems probable from the *in vivo* experiments of Wiechowski, our present conceptions of gout and purine metabolism must be much modified. The tissues of the human fetus at various stages of development show no more evidence of uricolysis than do the tissues of the adult. It is impossible to explain the positive results reported by Schittenhelm and Schmidt

with the tissues of the infants at or near term, but in view of the unanimity of opinion as to the absence of uricolitic activity in adult tissues, their results are *a priori* doubtful. We cannot, therefore, explain the presence of urate deposits in the kidney of new-born infants as due to tardy development of uricolytic enzymes in the human fetus, as suggested by the results obtained by Mendel and Mitchell with pig embryos. More probably the cause of these deposits is simply the high proportion of uric acid in the urine of the new-born, as maintained by Sjöqvist, the deposition perhaps being favored by local conditions in the kidney, such as the hyaline matrix described by Flensburg. Our failure to find evidence of uricolytic activity in either fetus or placenta, together with the lack of uricolytic enzymes in human organs and tissues, throws doubt upon the statements in the older literature that allantoin is found in the urine of pregnant women and new-born infants, since so far as known allantoin is formed only through decomposition of uric acid.

As additional observations on the distribution and behavior of uricolytic enzymes, may be mentioned the finding of active uricolytic power in the liver of the guinea-pig; its absence in the organs of the turtle; its absence in the spleen, and probably also in the bone-marrow and leucocytes of the dog; and also the failure to demonstrate inhibition of uricolysis by serum (dog).

# ENZYMES OF TUBERCULOUS EXUDATES.

BY EUGENE L. OPIE AND BERTHA I. BARKER.

## ENZYMES OF TUBERCULOUS EXUDATES.<sup>1</sup>

By EUGENE L. OPIE AND BERTHA I. BARKER.

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New York.)

In a former publication<sup>2</sup> we have shown that tuberculous tissue contains an enzyme which digests protein with considerable activity in the presence of an acid medium. This enzyme is present in the tubercle at all stages of its development and disappears only after caseation has occurred. During the formation of the lesion polynuclear leucocytes are abundant and for the first weeks after inoculation the newly formed tissue exhibits the enzymotic activity characteristic of these cells, namely, an ability to digest protein in the presence of an alkaline medium. At the end of about four weeks polynuclear leucocytes have in great part disappeared and the tissue has almost wholly lost its power to digest protein in the presence of alkali. Since the tissue at this time consists almost wholly of mononuclear epithelioid cells it may be assumed that they contain the enzyme which digests in an acid or in an approximately neutral medium but fails to digest in the presence of an alkaline medium. The cells of the tubercle are similar in appearance to the large mononuclear phagocytes of an inflammatory exudate and to the phagocytic cells which are abundant in the sinuses of lymphatic glands in the neighborhood of a focus of inflammation, and all these cells cause digestion of protein under the same conditions. The enzyme of such cells differs from the autolytic enzyme of various organs, for example of the liver, in its greater activity.<sup>3</sup>

The demonstration of an active proteolytic enzyme in the epithelioid cells of the tubercle derives importance from the fact that these cells ingest and apparently dissolve tubercle bacilli and other

<sup>1</sup> Received for publication January 14, 1909.

<sup>2</sup> *Jour. of Exper. Med.*, 1908, x, 645.

<sup>3</sup> *Jour. of Exper. Med.*, 1906, viii, 410; and 1908, x, 645.

cells. It is noteworthy that Bergel<sup>4</sup> has recently obtained evidence that the same cells contain an enzyme which digest soft wax and such fatty substances as neutral butter fat and neat's-foot oil. The following observations which in large part confirm those of our previous study have been made during the course of experiments undertaken for another purpose.

The serum of the blood even in small quantity inhibits the action of the enzyme contained in the polynuclear leucocytes (leucoprotease) and this antienzymotic action is exerted as well by the serum of an inflammatory exudate. Our former study has shown that the serum of a tuberculous exudate obtained by injecting tubercle bacilli into the pleural cavity of the dog exhibits the same antienzymotic action upon leucoprotease. Moreover, the serum of the blood likewise inhibits that enzyme which, constantly present in tuberculous tissue, digests protein in the presence of acid, but experiments recorded in our former publication have shown that the power of the serum of the tuberculous exudate to inhibit the enzymes of tuberculous tissue may be much less than that of the blood. Nevertheless these experiments were inconclusive because the tuberculous tissue employed for the test was obtained only two weeks after inoculation at a time when polynuclear leucocytes were numerous and leucoprotease (inhibited by the serum) was present in abundance. Partial inhibition may have been referable to the action of the serum on leucoprotease. In the following experiment, although the tuberculous tissue employed exhibited some digestion in alkali (indicating the presence of leucoprotease), digestion in acid was predominant. The methods employed have been fully described in the article mentioned above.

EXPERIMENT I.—Tuberculous tissue from the mediastinum of a dog forty-two days after inoculation of the right pleural cavity with *Bacillus tuberculosis* (human), was passed through a miniature sausage machine and, suspended in salt solution with addition of toluol in small quantity, was allowed to stand in the ice chest. The fluid freed from large solid particles by filtration through gauze was incubated (5 c.c. being used for each test) during five days with coagulated protein in the presence of 0.2 per cent. acetic acid, 0.2 per cent. sodium carbonate and in approximately neutral medium (the volume of each mixture being 25 c.c.), in order to test its proteolytic activity.

<sup>4</sup> *Münchener med. Woch.*, 1900, lvi, 64.

The suspension of tuberculous tissue (enzyme) plus coagulated protein contains nitrogen in incoagulable substances represented by 2.6 c.c.

	0.5 per cent. Acetic acid.	Neutral.	0.5 per cent. Sodium carbonate
Extract of tuberculous tissue + coagulated protein after 5 days at 37° C.....	9.9	6.6	5.5
Digestion (control being 2.6).....	7.3	4.0	2.9

The same suspension of tuberculous tissue was allowed to act upon the same quantity of coagulated protein in the presence of various quantities both of blood serum and of serum from a tuberculous exudate removed from the pleural cavity of a dog forty-seven days after inoculation.

	After 5 days at 37° C.
Ext. tuberculous tissue + coag. protein,	6.6
Ext. tuberculous tissue + coag. protein + 2.5 exuded tuberculous serum,	9.85
Ext. tuberculous tissue + coag. protein + 1 exuded tuberculous serum,	7.55
Ext. tuberculous tissue + coag. protein + 0.5 exuded tuberculous serum,	6.35
Ext. tuberculous tissue + coag. protein + 0.25 exuded tuberculous serum,	5.3
Ext. tuberculous tissue + coag. protein + 0.1 exuded tuberculous serum,	4.0
Ext. tuberculous tissue + coag. protein + 2.5 blood serum,	3.9
Ext. tuberculous tissue + coag. protein + 1 blood serum,	3.55
Ext. tuberculous tissue + coag. protein + 0.5 blood serum,	3.5
Ext. tuberculous tissue + coag. protein + 0.25 blood serum,	3.45
Ext. tuberculous tissue + coag. protein + 0.1 blood serum,	3.4

The action of the exuded tuberculous serum (1 and 0.5 c.c.) employed in this experiment upon coagulated protein under conditions similar to those described above is shown by the following tests:

Exuded tuberculous serum (1 c.c.) plus coagulated protein contains nitrogen in incoagulable substances represented by 1.65 c.c. *N*/10 sulphuric acid (control).

	After 5 days at 37° C.	Digestion.
1 c.c. exuded tuberculous serum + coag. protein,	4.3	2.65
0.5 c.c. exuded tuberculous serum + coag. protein,	1.9	0.25

The foregoing experiment shows that the addition of 1 or 2.5 c.c. of exuded tuberculous serum not only fails to inhibit digestion by tuberculous tissue in a neutral medium but actually increases it. Digestion caused by this tuberculous tissue in a neutral medium is due to the action of two enzymes one of which digests in acid and neutral media but fails to digest in alkali (enzyme of epithelioid cells), whereas the other digests in alkaline and in neutral media but fails to digest in acid (leucoprotease). Inhibition of the latter

enzyme alone may explain the diminished digestion in the presence of very small quantities (0.1 to 0.5 c.c.) of exuded tuberculous serum—quantities too small to exhibit the proteolytic activity which is exerted by 1 c.c. but not by 0.5 c.c. of serum (see experiment).

In the experiments just described serum was separated from the cells of the exudate by centrifugalization. Since the cells, though scant in quantity, contain additional enzyme, the whole exudate will probably exert greater proteolytic activity than the serum alone. This suggestion is confirmed by the following test:

EXPERIMENT 2.—Turbid fluid was withdrawn from the right pleural cavity on the seventy-sixth day after inoculation with *Bacillus tuberculosis*.

	Control.	After 5 days at 37°.	Digestion.
1 c.c. tuberculous exudate + coag. protein,	1.2 (approx.)	7.25	6.05 (approx.)
1 c.c. ser. of tuberc. exudate + coag. protein,	1.2	4.85	3.65

The same test was repeated with exudate withdrawn two days latter.

	Control	After 5 days at 37°.	Digestion.
1 c.c. tuberculous exudate + coag. protein,	1.45	4.75	3.3
1 c.c. serum of tuberculous exudate + coag. protein,	1.2	3.05	1.85

In this experiment proteolytic action exerted by the whole exudate has been relatively weak because cells have been present in scant amount. Since the whole exudate digests with greater activity than its serum alone it is evident that the presence of serum increases the enzymotic action of the cells.

Unusual opportunity to study the proteolytic action of cells of a tuberculous exudate was afforded by a fluid removed from the pleural cavity fifty-seven days after intrapleural infection with a virulent (bovine) tubercle bacillus. The exudate which was small in amount was thick and resembled pus. Cells, in great part large mononuclear cells, were present in great abundance and serum was scant in amount.

EXPERIMENT 3.—Fifty-seven days after intrapleural inoculation with 1 c.c. of a translucent suspension of *Bacillus tuberculosis* (bovine) death has occurred and the pleural surfaces, mediastinum and adjacent lymphatic glands exhibit abundant newly formed tuberculous tissue which is in part caseous. The right pleural cavity contains 3 c.c. of thick yellow pus-like fluid; agar-agar inoculated with this fluid has remained sterile. Microscopic examination of the exudate

shows that it contains an immense number of cells. Large mononuclear cells with round oval or indented nuclei, and abundant protoplasm, form the greater bulk; these cells which resemble the epithelioid cells of tuberculous tissue in the dog often contain tubercle bacilli. Polynuclear leucocytes occur in large number and lymphocytes are numerous. It is not possible to determine the relative number of the different kinds of cells because the large mononuclear cells in considerable part occur in clumps, whereas the other cells are scattered.

Nitrogen of incoagulable substances in 0.25 c.c. exudate plus coagulated protein is represented by 2.4 c.c. *N*/10 sulphuric acid.

	Acetic acid. 0.2 per cent.	Neutral.	Carbonate 0.2 per cent.
0.25 c.c. tuberculous exudate + coag. protein			
after 5 days at 37° C.....	14.4	14.45	6.2
Digestion, .....	12.0	12.05	3.8

Whereas in Experiment 2, 1 c.c. of serous tuberculous exudate has caused digestion represented by 7.25 c.c. of *N*/10 sulphuric acid, in the foregoing experiment 0.25 c.c. of the tuberculous exudate (without addition of acid or of alkali) has caused digestion represented by 14 c.c. *N*/10 sulphuric acid. The polynuclear leucocytes of pus cause maximum digestion in the presence of alkali, but this pus-like exudate which consists in great part of large mononuclear cells, digests with far greater activity in a neutral or acid than in an alkaline medium. Nevertheless in the exudate used in Experiment 3 polynuclear leucocytes are present and there is referable to them some digestion in an alkaline medium.

Former experiments have shown that the enzyme of polynuclear leucocytes digests in neutral as well as in alkaline media. In the foregoing experiment digestion in neutral medium is referable to the two enzymes acting together. When allowance is made for this fact it is evident that digestion in acid caused by the mononuclear cells which are predominant must be more active in an acid than in neutral medium—a conclusion in accord with previous observations made with tuberculous tissue.

Our former study has shown that the serum of a pleural tuberculous exudate (obtained by experimental inoculation) unlike the serum of the blood or the serum of an inflammatory exudate obtained by intrapleural injection of a sterile irritant causes fairly active digestion of protein. An attempt has been made to determine the conditions which influence this exceptional proteolytic activity.

Fractional precipitation of the proteins of the blood serum have shown that the globulin contains an enzyme or combination of enzymes which is somewhat more active in a neutral than in an alkaline medium<sup>5</sup>; the albumin fraction contains anti-enzyme. In the whole serum the enzyme of the globulin is restrained by the anti-enzyme of the albumin, so that the serum of the blood when brought into contact with denaturalized protein fails to cause digestion. The enzymotic action of the tuberculous exudate digesting more energetically in neutral than in acid or alkaline medium resembles that of the globulin fraction of the blood. It is not improbable that proteolysis in both instances is brought about by a combination of two enzymes. Such a combination of two enzymes, one of which—leuco-protease—digests in acid and neutral media, whereas another, *e. g.*, the enzyme of tuberculous tissue, digests in neutral and acid media may produce greater digestion in neutral than in either acid or alkaline medium. Of essential importance in the present study is the observation that enzymes may exist in tuberculous exudate unrestrained by anti-enzyme.

A considerable number of observations have been tabulated (Table I) in order to show the relationship of the enzymotic activity of exuded tuberculous serum to the duration and course of the infection, and to inoculation with different strains of tubercle bacilli (human and bovine).

In every instance one cubic centimeter of exuded serum obtained by centrifugalization of the tuberculous exudate has been allowed to act upon five cubic centimeters of coagulated protein during five days at 37° C. The figures in the table represent the amount of digestion which has occurred, and have been obtained by subtracting the figure representing the control, *i. e.*, incoagulable nitrogen in the mixture before incubation, from the figure representing incoagulable nitrogen after incubation during five days at 37° C.

With the progress of the infection, following inoculation with the human type of bacillus there is no increase of proteolytic activity; there is in general a slight decrease. This observation is contrary to the impression mentioned in our former publication on the subject.

<sup>5</sup> Opie and Barker, *Jour. of Exper. Med.*, 1907, ix, 207.



In two instances serum of exudates obtained by inoculation with the human type of bacillus failed to cause noteworthy digestion. In one such instance fluid was removed after death; in the second the animal had lost much weight and was very sick.

In both instances in which the bovine type of tubercle bacillus has been used there is an almost complete absence of proteolysis, whereas animals inoculated with human tubercle bacilli have shown fairly active proteolysis at corresponding periods after inoculation. It is not improbable that this difference bears some relation to the virulence of the two strains, for the bovine type kills dogs with much greater certainty and after a shorter interval.

Attempts have been made to determine if the serum of human tuberculous exudates causes proteolysis similar to that obtained after inoculation of dogs. Such tests have been uniformly negative. The following examples are cited from a number of almost identical observations for which we are indebted to Dr. A. R. Dochez.

*Tuberculous Pleurisy.*—McC., Presbyterian Hospital of New York. Yellowish slightly turbid fluid was withdrawn October 21 from the pleural cavity. Serum was obtained by centrifugalization. No bacteria were grown from the fluid; tubercle bacilli were not found. Clinical diagnosis is tuberculous pleurisy.

	Control.	After 5 days at 37°C.
1 c.c. exuded tuberculous (?) serum + coag. protein,	1.5	1.7
2.5 c.c. exuded tuberculous (?) serum + coag. protein,	1.8	1.9

*Tuberculous Pleurisy.*—Q., Presbyterian Hospital. Coagulable fluid was withdrawn from the pleural cavity; a guinea-pig inoculated with this fluid died with generalized tuberculosis. Serum obtained by centrifugalization of the pleural exudate gave the following result, tests being made in duplicate:

	Control.	After 5 days at 37°C.
1 c.c. exuded tuberculous serum + coag. protein,	2.1	2.3
		1.9
2.5 c.c. exuded tuberculous serum + coag. protein,	2.2	2.4
		2.35

*Tuberculous Peritonitis.*—H. G., Presbyterian Hospital. Slightly turbid orange yellow fluid was removed by tapping from the peritoneal cavity; a small quantity of blood was present and a coagulum which formed was somewhat blood stained.

	Control.	After 5 days at 37°C.
1 c.c. exuded tuberculous serum + coag. protein,	1.6	1.8
2.5 c.c. exuded tuberculous serum + coag. protein,	2.25	2.65

At autopsy the peritoneal cavity contained 150 c.c. of yellow fluid; the omentum was thickened, retracted and partly caseous. The peritoneal surfaces were studded with large tubercles, undergoing caseation.

The observations which have been described confirm those previously recorded and show that the mononuclear epithelioid cells of tuberculous tissue contain an enzyme which digests protein actively in an acid and with slightly less rapidity in an approximately neutral medium, but is almost wholly inactive in the presence of alkali. This enzyme unlike the enzyme of the polynuclear leucocytes is not inhibited by the serum of a tuberculous exudate obtained by injection of tubercle bacilli (human) into the pleural cavity of the dog, although it is inhibited by the blood serum. The serum of such an exudate, moreover, unlike the serum of the blood is capable of digesting denaturalized protein. Two observations indicate that this proteolytic activity may disappear just before death. Such proteolytic activity is exhibited by exuded serum of animals inoculated with an organism to which they are relatively insusceptible, and is absent in animals inoculated with a more virulent type (bovine) of tubercle bacillus. Power to digest protein has not been demonstrable in the serum of human tuberculous exudates.





## EXPERIMENTAL STUDIES ON PNEUMOCOCCUS INFECTIONS.<sup>1</sup>

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The inefficiency of specific sera in the treatment of pneumonia must be attributed in part at least to the lack of exact knowledge of the nature of the infection and the processes whereby the human organism protects itself. To ascertain detailed facts regarding pneumococcus infection one must resort to laboratory animals, none of which so far as is known reacts to such infections in identically the same manner as does man.

At the suggestion of Dr. Rufus Cole, to whom I am greatly indebted for much assistance and encouragement, I undertook the study of the immunity of the pigeon, but very early in the work it was found that to understand the reaction of the immune animal correctly it would be necessary to have exact knowledge of the reaction of the susceptible animal under identical conditions. A considerable part of this work was directed to the study of leucocytosis and phagocytosis, especially in their relations to virulence of pneumococci.

One interesting theory of the nature of the crisis is based on phagocytosis in the lung (Tchistovitch) (1). Although Mennes (2) in 1897 found that active immunity in rabbits to pneumococcus infection was due to a modification of the serum which allowed of phagocytosis, rather than to a specific change in the property of the leucocytes, Heim (3) in 1908 observed that the bone-marrow of immunized rabbits had two and a half times as much protective power as did serum. Rosenow (4) in 1906 claimed to have shown an increased resistance to heat and an increased phagocytic power of

<sup>1</sup> Aided by a grant from the Rockefeller Institute for Medical Research. Received for publication July 1, 1909.

pneumonic leucocytes for pneumococci, and in the recent contributions of Hiss (5) to the study of leucocyte extracts mention is made of an observation by North that the leucocytes of a pneumonia patient were more actively phagocytic than those of a normal man. The older investigations of Tchistovitch (1) on pneumonia had led him to believe that susceptibility and immunity to pneumococcus infections were to be measured by the degree of phagocytosis of which the leucocytes of the animal under study were capable.

In this work we used pneumococci obtained from the blood of patients suffering from pneumonia, and as numerous cultures were made we had the opportunity of studying numerous strains of freshly isolated bacteria as well as older cultures. Unless especially stated in the protocols, twenty-four cultures on human blood agar slants were used. The animal injections were always given intraperitoneally. All experiments were carefully controlled, and repeated until there could be no doubt as to the results. It would, however, be a waste of space to attempt to give tables of all the experiments, and I have, therefore, reproduced below only typical charts, or, where such seemed better to serve the purpose, composites.

#### EXPERIMENTS IN PHAGOCYTOSIS.

The technique employed in studying phagocytosis *in vitro* was the usual Wright capillary pipette method, with incubation at 37° C. for fifteen minutes. Rather thick suspensions of bacteria in salt solution of as uniform a consistency as possible were used. The usual counting method of estimating opsonic index was not employed, as we believe that the errors inherent to such a method are as great as, or greater than, those included in broad standards of phagocytosis like "slight," "active," "marked."

It is a matter of considerable service to have a handy index of virulence. Several workers, especially Rosenow (4) and Graham (6) claim that phagocytability as tested by normal serum plus corpuscles with the Wright technique is an excellent index of virulence. We made numerous tests to prove this point. The method used was to study strains of pneumococci by parallel opsonic tests and peritoneal injections into white mice, following the course of the latter injection by withdrawing the fluid at various times.

TABLE I.

Culture.	Phagocytosis <i>in vitro</i> .	Phagocytosis in mouse.	Result in mouse.
P <sub>2</sub>	0	0	Death
P <sub>3</sub>	+	+	Lived
2D	+	+	Lived
2K	0	0	Death
P <sub>4</sub>	0	0	Death
P <sub>10</sub>	±	±	Death
P <sub>12</sub>	0	0	Death
P <sub>5</sub>	±	±	Varied

Our experiments, which are grouped in Table I, showed that there is a direct parallelism between phagocytosis *in vitro* and *in vivo*. An organism which is not taken up by normal human cells in a capillary pipette will not be susceptible to phagocytosis in the body of a mouse. Further, although there are other complicated factors bearing on virulence, these results show that "insusceptibility to phagocytosis" and "virulence" are closely related, and broadly speaking, a phagocytable organism may be called "avirulent" and a non-phagocytable one "virulent."

A study of virulence of pneumococci would not be complete without mention of the work of Rosenow (7) on "Virulin." By suspending pneumococci in physiological salt solution for forty-eight hours, he was able to extract from the cocci a substance which when added to blood serum has the faculty of combining with or neutralizing the pneumococcus opsonins. This so-called "virulin" likewise has the power of combining with a phagocytable coccus and rendering it non-phagocytable, while the original organism after treatment when suspended in normal serum is taken up by leucocytes. This salt autolysate of pneumococci was one year later described by Tchistovitch and Yourevitch (8) as "antiphagin." We tested the effect of virulin extracted according to Rosenow's direction from four different virulent strains of pneumococci on five other strains of avirulent organisms and were unable to perceive any change in the phagocytability of these cultures after suspension. As Dr. J. A. Leutscher working in this laboratory undertook to study this interesting question more extensively and will soon report his results, further details of my experiments need not be mentioned.

## THE INFECTION IN THE WHITE MOUSE.

The technique employed in all animal experiments was identical. At stated times after intraperitoneal injections, fluid is removed by pipettes through the abdominal wall as in the study of the Pfeiffer phenomenon. Smears made at once were stained with carbol-thionin.

The first preliminary experiments seemed to indicate that avirulent bacteria caused a marked outpouring of leucocytes, whereas the injection of virulent ones was followed by only a few leucocytes. But numerous further observations showed that the leucocytic reaction following pneumococcus injections is practically the same whether the organism is virulent or avirulent. The initial presence of small mononuclears and the appearance later of polymorphonuclears and macrophages is characteristic of almost any intra-peritoneal injection, as is seen in Table II, composed of the results of several experiments.

TABLE II.

Dose.	P <sub>0</sub> 4 oese.					P <sub>0</sub> 1 oese					sD 1 tube.				P <sub>0</sub> 1 oese.					Peptone 2 c.c.			
Hours.	1	3	5	24	48	1	3	5	24	48	1	3	5	24	1	3	5	24	56	1	3	5	24
Leucocytes (polymorphonuclears)	+	+	+	+		+	+	+	+		+	+	+	+	+	+	+	+		+	+	+	+
Phagocytosis.	o	+	+	o		o	o	o	o		+	+	+	+	o	o	o	o					
Number of free pneumococci.	+	+	+	+	Death.	+	+	+	+	Death.	+	+	+	+	o	Lived.	+	+	+	+	Death.		

Table II also shows that the organism which is not phagocyted kills the mouse, whereas the phagocytable strain is harmless. However, it is not uncommon to find that one of two non-phagocytable organisms will kill, while the other in the same dose does no apparent damage, and it is impossible to forecast what the minimal lethal dose of either will be. Moreover, if the ability to withstand phagocytosis were the only element in virulence, it would be extremely difficult to understand the fact which Marchand (9) found for streptococci and which I have abundantly verified for

numerous strains of pneumococci, namely, that even after heating to 60° C. for one hour and washing, an organism which is not phagocytable before the procedure will not be phagocytable after treatment, nor can any difference be seen in the cellular outpouring following injection of either dead or living cultures. The injection of living organisms produces death of the animal, and the injection of the dead culture does no harm. This difference is undoubtedly due to the ability of the living organisms to grow. Many observations on injections of small doses of non-phagocytable pneumococci which do not kill the animal show that their harmlessness is associated with inability to grow in the mouse's peritoneal cavity. Changing the point of view to the last, this is equivalent to saying that the peritoneal cavity of even the most susceptible animal possesses the property of destroying certain numbers of pneumococci; and the result of a pneumococcus inoculation will depend on the bactericidal as well as on the phagocytic powers of the animal.

To study further the effect of leucocytes on pneumococci, sterile peritonitis was produced by peptone injections, and pneumococcus inoculations made approximately three hours later. The peritoneal reaction did not differ from that seen after simple pneumococcus infection nor were mice protected by this procedure.

#### THE "IMMUNITY" OF THE PIGEON.

The pigeon offers itself as the best subject for the study of "natural immunity," as it is absolutely insusceptible to infection by the pneumococcus. The first line of work undertaken was a comparative investigation of pigeon serum and leucocytes from the standpoint of phagocytosis. Leucocytes were obtained either by aspiration from a vein or from the peritoneal cavity after injection of peptone, and their phagocytic power tested by the Wright method. The results of these experiments are recorded in Table III.

This chart shows that *in vitro* pigeon corpuscles and serum behave toward the pneumococcus in no way differently from those of normal man. Certain unrecorded experiments of Dr. Cole (personal communication) gave the same results. One cannot, however, conclude from these tests what results *in vivo* might be, and we, therefore, considered it a fruitful investigation to study the

TABLE III.

Leucocytes.	Serum.	Culture.	Phagocytosis.	Remarks.
Human.	Human.	P <sub>2</sub>	Slight.	
Pigeon.	Pigeon.	P <sub>2</sub>	o	
Human.	Human.	2K	o	
Pigeon.	Pigeon.	2K	o	
Pigeon peritoneal.	Pigeon periton.	2K	o	
" "	" blood.	2K	o	
" blood.	" "	2K	o	
" "	" periton.	2K	o	
" peritoneal.	" "	2K	o	40 minutes
" "	" "	2K	o	1 hour.
Human.	Human.	3A	++	
Pigeon.	Pigeon.	3A	++	
" (defibrinated)	"	3A	++	
Human.	Human.	R	o	
" "	"	F	o	
" "	"	C	o	
" "	"	D	++	
Pigeon.	"	R	o	
" "	"	F	o	
" "	"	C	o	
" "	"	D	++	
Human.	Pigeon.	R	o	
" "	"	F	o	
" "	"	C	o	
" "	"	D	++	
Pigeon.	"	R	o	
" "	"	F	o	
" "	"	C	o	
" "	"	D	+	

reaction in the peritoneal cavity in the same way as was done with the white mouse.

The observations in detail, with varying doses of numerous strains, showed that the peritoneal reaction scarcely differed from that of the mouse, except that the polymorphonuclear leucocytes possessed large eosinophilic granules. Phagocytosis was no more marked than in the mouse, but in every case, generally within three to five hours, there was complete disappearance of the organisms, no matter how virulent they may have been for the mouse. In no case could we kill a pigeon, however large the dose.

To study the possible causes of this disappearance, fourteen pigeons were inoculated with a slightly phagocytatable organism and were killed at intervals of one-half hour. Smears from the heart, liver and peritoneum, and cultures from the heart were made in every case. No pneumococci were demonstrated in smears from the heart or liver. Cultures from the heart gave pneumococci in

two instances, twenty minutes and two and a half hours after injection.

The peritoneal reaction may be summarized in the following statements:

1. A gradual outpouring of cells as in the mouse, with development of general peritonitis in about three hours. Phagocytosis is slight, the englobed cocci showing no capsules, nor are capsules seen in those cocci within an annular zone around each leucocyte.

2. Despite lack of very active phagocytosis, the pneumococci gradually disappear completely. There is no agglutination, and no bacterial degenerations suggesting lysis.

The organism used in this experiment took a capsule stain very easily and the lack of capsules in those bacteria immediately around polymorphonuclear leucocytes as well as in those already ingested was striking. This fact seems to indicate that virulence or at least phagocytosis can have no direct dependence on capsule formation, for in other experiments organisms which take capsule stain only with difficulty are not phagocyted at all. On the whole phagocytosis was so slight that one was forced to doubt if the function of the pigeon polymorphonuclear leucocyte is truly phagocytizing, and several experiments were, therefore, undertaken to test this. Comparative observations were made with virulent pneumococci, phagocytatable pneumococci and staphylococci as shown in Table IV.

TABLE IV.

Culture.	Virulent pneumococcus.				Avirulent pneumococcus.				Staphylococcus.			
	1	2	3	4	1	2	3	4	1	2	3	4
Hours .....												
Leucocytes .....	+	+	+	+	+	+	+	+	+	+	+	+
			+	+			+	+			+	+
				+				+				+
Phagocytosis .....	o	o	o	o	o	+	+	+	+	+	+	+
Number of free pneumococci .....	+	+	±	o	+	+	±	o	+	+	+	±
	+				+				+			

Table IV shows that pigeon leucocytes are well able to phagocyte staphylococci and that they may take up pneumococci with about the same or perhaps less avidity than do the leucocytes of a mouse.

Although Tchistovitch (10) claims to have found that certain of the pigeon polymorphonuclears act as phagocytes, while others do not, we were unable to demonstrate any differences beyond those seen in all observations on phagocytosis. Some of the polymorphonuclear leucocytes in the mouse peritoneum were loaded with cocci, whereas others were free; and such appearances are not uncommon in opsonic smears or in gonorrhoeal pus in man.

Although our observations indicate that phagocytosis plays an insignificant rôle in the destruction of the pneumococci in the pigeon, it must nevertheless be borne in mind that possibly phagocytosis may occur associated with such rapid digestion of the ingested bacteria as to render them unable to take up stains. The fact that "avirulent" organisms are well stained inside leucocytes weighs against this supposition, but is not definite proof. The very increased virulence of the pneumococci may render them capable of being rapidly digested by leucocytes, just as it seems to make them susceptible to rapid lysis by bile, as has been recently shown by Neufeld (11) and Grixoni (12). Furthermore Neufeld (13) finds that agglutination occurs more rapidly with virulent than with avirulent strains of pneumococci.

This theoretical possibility admits of practical tests by experiments on the effect on pneumococci *in vitro* of pigeon peritoneal exudate rich in leucocytes. Injections of a saturated solution of peptone in water or bouillon will produce an exudate indistinguishable from that caused by pneumococci. The cultures used were twenty-four hour blood agar slants of both avirulent and virulent organisms, finely emulsified in bouillon and allowed to stand for an hour until the coarser clumps settled. One part of culture was used to two parts of the exudate to be tested, carefully measured. Controls were made by mixing bouillon and culture in the same proportions. The exudate alone was also plated. After remaining in the thermostat at 37° C. for varying times, as shown in Table V, agar plates were made by mixing one standard loop with 10 c.c. of agar. Colonies from the plates were examined and identified as pneumococci. Bearing in mind the rapid and complete destruction of the pneumococcus in the pigeon's peritoneal cavity and the natural unstable viability of the pneumococcus, it was determined

to adopt as a standard only changes in the colony count marked enough to be seen with the naked eye. The figures in the charts represent the number of colonies counted in ten fields chosen at random under the low power ( $3 \times 2/3$ ) and correspond well with the naked eye appearance. The results of all experiments agreed with those expressed in the table in showing that fresh pigeon peritoneal exude has no bactericidal effect on pneumococci *in vitro* at 37 degrees C.

TABLE V.

	C.c.	Colonies in ten microscopic fields.		
		One-half hour.	Two hours.	Twenty hours.
Pigeon peritoneal exudate.....	0.5			
Pneumococcus 8.....	0.25	7	7	$\infty$
Pigeon peritoneal exudate.....	0.5			
Pneumococcus 11.....	0.25	6	0	15
Pigeon peritoneal exudate.....		0	0	0
Bouillon .....	0.5			
Pneumococcus 8.....	0.25	14	15	3
Bouillon .....	0.5			
Pneumococcus 11.....	0.25	14	18	0

The use of bouillon might be objected to on the ground that it is a poor culture medium for the organism we are testing. We, therefore, made a series of observations with eighteen hour litmus milk cultures, milk being an unusually good soil for growth of pneumococci. The results in this series were also constant and may be seen in Table VI.

TABLE VI.

*Milk Cultures of Pneumococcus.*

	C.c.	One-half hour.	Two hours.	Twenty hours.
Pigeon peritoneal exudate.....	0.4	++	$\infty$	0
Pneumococcus 8.....	0.2			Very acid.
Pigeon peritoneal exudate.....	0.4	+	0	0
Pneumococcus 10.....	0.2			Very acid.
Pigeon peritoneal exudate.....		0	0	0
				Alkaline.
Bouillon .....	0.4	+++	$\infty$	0
Pneumococcus 8.....	0.2			Acid.
Bouillon .....	0.4	0	0	0
Pneumococcus 10.....	0.2			Acid.

Although this chart seems to show a marked lack of growth of the pneumococci in both the tests and the controls, further consideration disproves this appearance. At the end of twenty hours all the tubes were acid, those containing the bacteria plus exudate being considerably more so than the controls. On smears large numbers of well stained diplococci were seen. We had often found that pneumococci which grow well in milk might not grow when transferred to the other media, and we believe that this is what occurred in these experiments. Mixtures of peritoneal exudate and sterile litmus milk will remain alkaline, as I was able to show; and consequently the greater acid production seen in the mixtures of exudate and pneumococcus culture, as compared with the controls, indicates that the organism grows better in the presence of this exudate than it does in bouillon.

That powerful bactericidal substances can be extracted from the leucocytes by various means is well known. Hiss (5) was able by mere extraction with sterile water to produce bodies with a marked protective power over infections by various bacteria including the pneumococcus. This body, he concludes, has the property of neutralizing bacterial endo-toxins. The bactericidal effect of extracts made according to Hiss's directions was tested in the same way as was the fresh exudate, and it was found that they had no effect on the growth of pneumococci, as is seen from Table VII, which is a composite of twelve observations.

TABLE VII.

	C.c.	Colonies in ten microscopic fields.		
		One hour.	Five hours	Twenty hours.
Leucocyte extract.....	0.2	∞		
Pneumococcus 12, in bouillon...	0.1		+++	10
Leucocyte extract. ....	0.2			
Pneumococcus 8, milk culture...	0.1	∞	+++	0, very acid.
Bouillon . ....	0.2			
Pneumococcus 12, in bouillon...	0.1	∞	+++	3
Bouillon ..... ..	0.2			
Pneumococcus 8, milk culture...	0.1	∞	+++	0
Leucocyte extract. ....	0.2	0	0	0
Sterile milk., ... ..	0.1			

The remarks made above in discussing Tables V and VI apply with equal force here.

Although these observations prove conclusively that pigeon exudate has no inhibiting action on the growth of pneumococcus *in vitro* at 37° C., it is necessary to test its power of combating the infection in the susceptible animal. White mice were given intra-peritoneal inoculations and autopsies were performed on all animals, and except where especially noted, showed nothing striking. In Table VIII each experimental mouse was given 1 c.c. of fresh peritoneal exudate, and the controls of 1 c.c. of physiological salt solution with the pneumococcus injection.

TABLE VIII.

Experiment.	Dose.	Result.	Pulmonary involvement.
1	T/10	Died 4 days.	
Control.	T/10	Lived.	
2	T/10	Died 42 hours.	R. up. and mid. lobes.
Control.	T/10	Lived.	
3	T/5	Died 18 hours.	
Control.	T/5	Died 18 hours.	
4	T/3	Lived.	
Control.	T/3	Lived.	
5	T/5	Lived.	
Control.	T/5	Died 18 hours.	R. mid. lobe.
6	T/5	Died 16 hours.	R. mid. lobe.
Control.	T/5	Died 8 days.	
7	T/6	Died 24 hours.	Both mid. lobes.
Control.	T/6	Lived.	
8	T/8	Died 24 hours.	Both mid. lobes.
Control.	T/8	Lived.	
9	T/5	Died 4 days.	
Control.	T/5	Lived.	

The experiments with watery extracts of pigeon leucocytes, obtained and washed as previously outlined, were not very satisfactory. The results were not definite and extended observations were not undertaken, inasmuch as Hiss's results demonstrate clearly that the action of such extracts is not specific. The results are seen in Table IX.

TABLE IX.

Experiment.	Dose.	Result.	Experiment.	Dose.	Result.
1	T/5	Died 10 days.	7	T/5	Lived.
Control.	T/5	Died 4 days.	Control.	T/5	Died 18 hours.
2	T/2	Died 3 days.	8	T/3	Lived.
Control.	T/2	Died 3 days.	Control.	T/3	Lived.
3	T/10	Died 3 days.	9	T/5	Died 8 days.
Control.	T/10	Died 24 hours.	Control.	T/5	Died 18 hours.
4	T/10	Lived.	10	T/5	Died 18 hours.
Control.	T/10	Lived.	Control.	T/5	Died 8 days.
5	T/15	Died 24 hours.	11	T/10	Lived.
Control.	T/15	Died 18 hours.	Control.	T/10	Lived.
6	T/10	Lived.	12	T/8	Died 18 hours.
Control.	T/10	Lived.	Control.	T/8	Lived.

These experiments are interesting from two points of view. Of nine mice injected with fresh pigeon exudate, five died while the control lived and one died in sixteen hours compared to eight days for the control. One survived a dose that killed the control in eighteen hours; and in the two remaining experiments there was no difference between the control and the animal under experiment. The experiments with washed and autolyzed peritoneal leucocytes resulted differently. In four cases both animals survived; in one, both died; five lived longer than the controls and two died earlier. The contrast is rather striking. In the first series in which the exudate is used the majority die earlier than the controls. In the second series the life of the majority is prolonged. This then shows a definite "pro-infective" action of the whole peritoneal exudate which is lacking when it is washed, and which seems to us must be due to the presence of serum in the whole fluid. Pettersen (14) noticed that leucocytic extract in bouillon possessed greater bactericidal powers than the same extract in serum, and Korschun (15) was able practically to inhibit the bactericidal effect of leucocytic extracts by the addition of inactivated normal serum. Recently Cole and Smirnow (16) have called attention to the "pro-infective" power of blood serum; animals given simultaneous inoculations of pneumococci and pigeon or rabbit serum die earlier than controls given the culture and physiological salt solution. White and Graham (17) consider the phenomenon a "sensitizing reaction," and find that it may be accomplished by very minute

quantities (.1 c.c.) of serum. Probably sufficient serum is present in the exudate removed from the peritoneal cavity of pigeons to account for its "pro-infective" action.

The incidence of pulmonary involvement in the first series of mice is interesting. In the gross, there was a definite lobar involvement; the infected lobes were dark red or dull grey in contrast with the normal pink lung tissue; some sank in water, others did not.

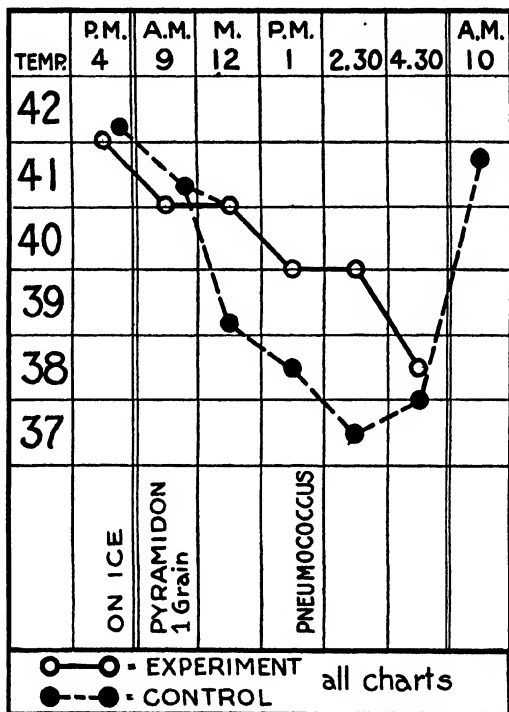


CHART I. Injected pigeon died during night; control lived. (See text.)

When the lungs were studied microscopically, however, in no case was lobar pneumonia seen, but simply very extensive broncho-pneumonia. It has been extremely rare to find lung involvement following injections of pneumococci in mice. No explanation is offered for the high percentage occurring with this particular method of injection.

Only brief mention will be made of attempts to cause pneumo-

coccus infections by combined injections in various ways of morphine, lactic acid and the filtrate of very old typhoid cultures with pneumococci. These all resulted negatively and the peritoneal reaction was that seen after ordinary pneumococcus injection. The alkalinity of the serum of a pigeon titrated against dimethylamidoazobenzol was practically the same as that of man.

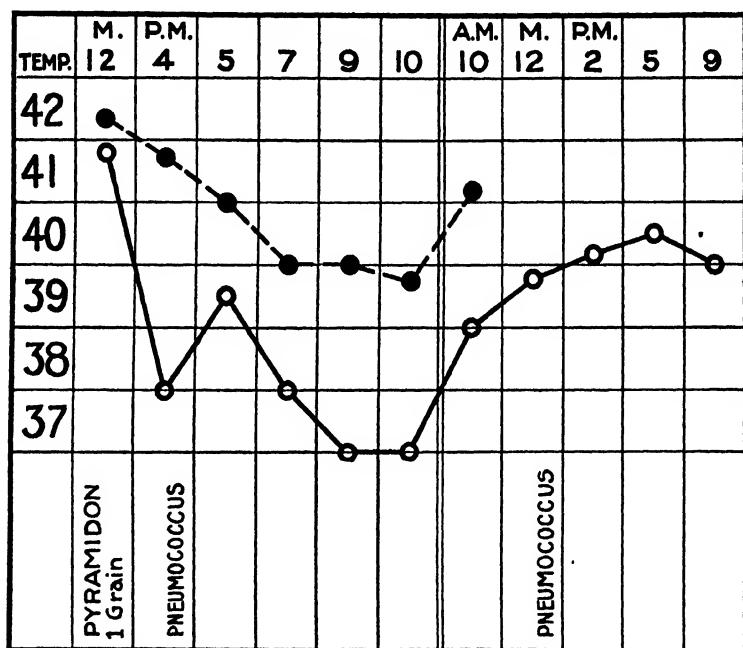


CHART II. Injected pigeon died 9 p. m.; control lived.

As the biological reactions in the pigeon to pneumococcus do not seem to differ from those in the mouse and cannot explain the difference in effects, it seemed wise to investigate any individual peculiarities of the pigeon.

The most striking difference between the pigeon and the white mouse or man is in the temperature, which in a series of thirty birds was found to average  $41.5^{\circ}$  ( $106.7^{\circ}$  F.). Tests on the thermal death point of the pneumococcus revealed the striking fact that between forty and forty-one there was inhibition of growth on human blood agar or in the exudate from the pigeon's peritoneal

cavity. In milk the cultures withstood a temperature of  $41.5^{\circ}$  C. These tests were made with numerous strains of the organism, and while a sharp thermal death point could not be demonstrated for all strains there was nevertheless a general inhibition of growth between the points named.

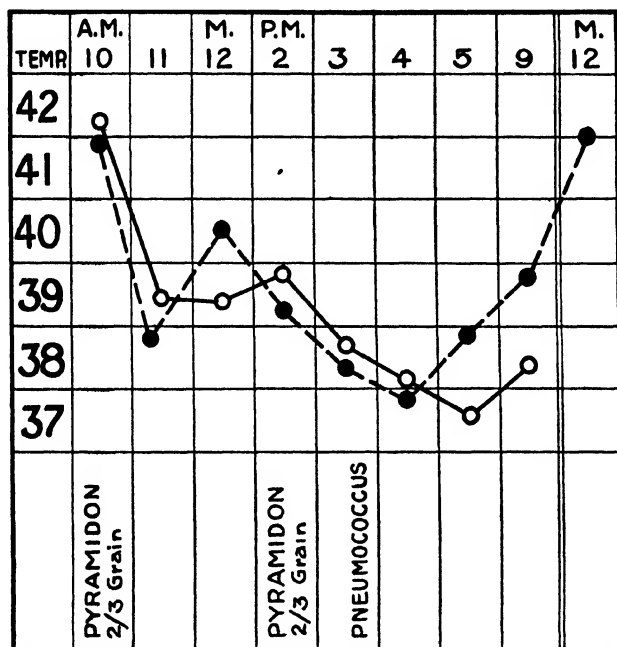


CHART III. Injected pigeon died during night; control lived.

It seemed reasonable to suppose that were it possible to reduce the temperature of the pigeon sufficiently, a pneumococcus injection would give the same results as occur in the mouse. Attempts to reduce the temperature according to the classical experiment of Pasteur by keeping the bird for twenty-four or forty-eight hours in melting ice caused no change. Subcutaneous injection of pyramidon (dimethylamidoantipyrin) was found to reduce the pigeon's temperature, and tests of its toxicity were made on sixteen pigeons, with the results seen in the control curves on the charts. Two pigeons, however, died within ten minutes of the injection of one grain, with symptoms resembling cerebellar ataxia, but in both

animals the injection had been made intramuscularly instead of subcutaneously, as was done in all other experiments. As the action of pyramidon is not very lasting, the organism used in the injection should be as virulent as possible, but at the time the experiments were performed it was impossible to procure such a culture, and we had to resort to large doses of a phagocyttable, rather avirulent, organism. Charts I to IV show conclusively that one can cause a pneumococcus septicaemia in the pigeon after its temperature has been lowered sufficiently. Charts V and VI are controls showing

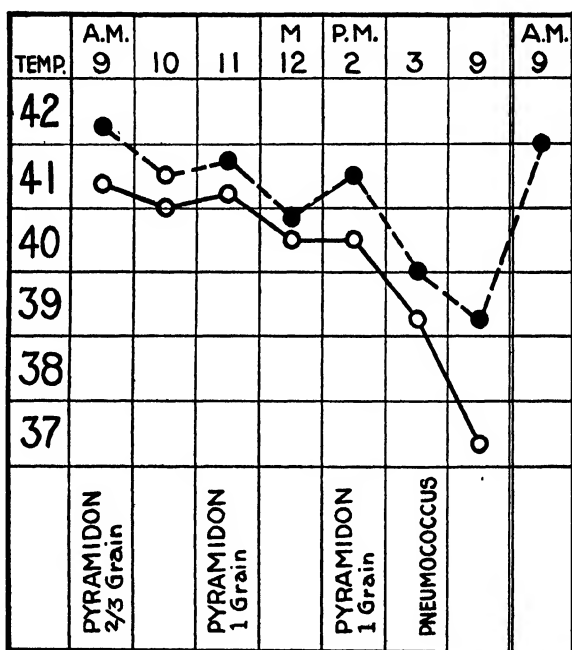


CHART IV. Injected pigeon died 9 p. m.; control lived.

that the mere combination of pyramidon and pneumococcus without reduction in the temperature will not give the same results as are seen when the temperature is lowered, and the control curves show that mere reduction of temperature is not sufficient to cause death. The control birds were kept under identically the same conditions as were the experimental birds.

In the charts the circles and unbroken lines represent the rectal

temperature of the experimental pigeons, while the solid dots and broken lines represent the controls. The treatment of the two birds was the same up to the injection of the pneumococcus, and here the control was given the same volume of sterile broth. No control died. Chart IV represents two experiments, the course of which was the same.

That death was due to the injection of pneumococci is clear from the following evidence. The pigeons receiving pneumococci were always sicker than the controls. Smears of the peritoneal fluid con-

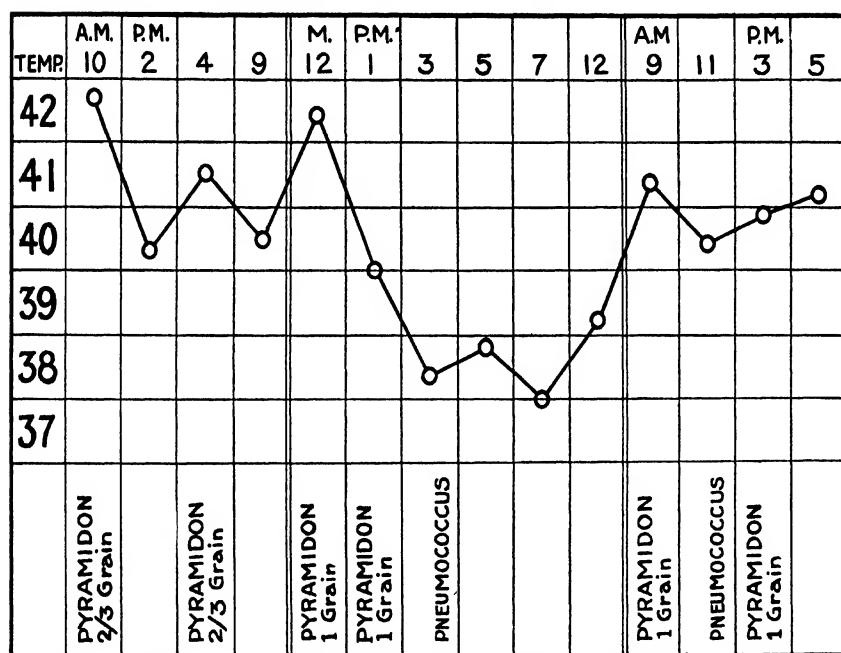


CHART V. Pigeon survived five injections of pyramidon and two of pneumococci.

tained free cocci as late as twenty hours after the injection, and the number of free organisms seemed to vary directly with the intensity of the symptoms. In pneumococcus injections at the pigeon's normal temperature the bacteria disappear in from three to eight hours, and the bird is usually no sicker than a control given any sterile fluid. Furthermore, autopsies were performed on all the birds. The peritoneum was inflamed, contained fibrin and free

fluid, which despite rather active phagocytosis showed in all cases large numbers of free diplococci. In three of the five pigeons it was practically impossible to distinguish the peritoneal fluid from that of a mouse dead of pneumococcus peritonitis. Cultures from the peritoneal cavity gave in all cases pure pneumococcus. Although

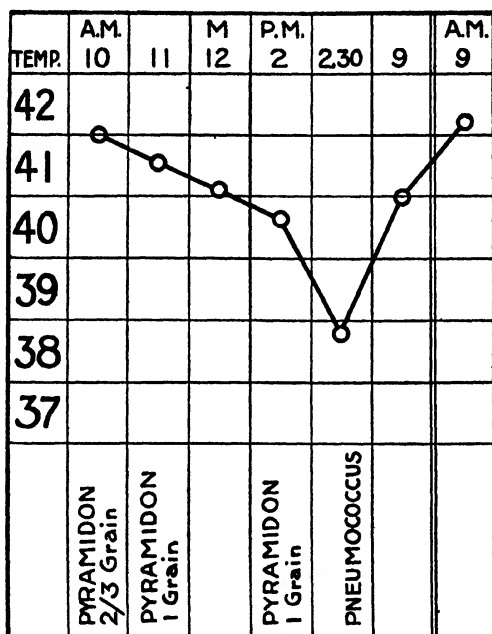


CHART VI. Pigeon survived.

smears from the heart's blood showed no organisms, cultures were positive in four cases, and in the fifth, the pneumococci were so abundant in the peritoneal cavity that the cause of death could not be doubted. Post-mortem growth and invasion of the blood stream is practically excluded by the fact that the pigeons were placed in the ice-chest almost immediately (one or two hours) after death.

#### SUMMARY.

1. Phagocytosis of pneumococci *in vitro* runs parallel with phagocytosis *in vivo*.
2. Virulence depends not only on resistance to phagocytosis, but also on the ability to grow in the body of the animal.

3. The biological reaction of the pigeon to pneumococcus infection does not differ from that of the mouse.

4. The "immunity" of the pigeon to pneumococcus infection is due to its normal high temperature.

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## ON AUTO-ANTIBODY FORMATION AND ANTIHEMOLYSIS.<sup>1</sup>

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### INTRODUCTION.

While a great deal has been learned within recent years regarding the defensive reaction on the part of the body against foreign cells and foreign cell products, introduced from without, our knowledge of the reactions of the body against its own cell derivatives, and as a matter of fact against its own cells is as yet very meager. I use the expression "against its own cells" advisedly, as there is abundant evidence to show that the body actually possesses forces which control not only normal, but also abnormal cell proliferation. In studying the protective mechanism of the animal body against its own cells and cell derivatives we naturally look for the same or similar methods by which it reacts to the introduction of foreign cells, and we may accordingly consider the possibility of auto-phagocytosis and auto-antibody formation. Both indeed seem to occur. From the evidence at hand, however, it does not appear probable that auto-phagocytosis represents an important method of defence, for in those cases in which it is observed the process seems purposeless, and rather a secondary result, the outcome of some other process which itself may indeed be obscured. I have thus noted phagocytosis of red cells by large mononuclear leucocytes in two cases of epidemic cerebro-spinal meningitis, where the corresponding organisms could be directly demonstrated in the circulating blood. In both cases

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many of the large mononuclear elements were engaged in the phagocytosis of diplococci and one gained the impression that the occasional phagocytosis of red cells were accidental rather than purposeful (1).

Mary W. Rowley (2) has reported a remarkable instance of auto-phagocytosis in a peculiar case of anemia in which all types of leucocytes were found to be phagocytic for both red and white cells, but in which large mononuclear elements also seemed to be especially active. It is noteworthy that the addition of the patient's blood to normal blood incited phagocytosis on the part of all the mononuclear cells of the mixed blood and that the subcutaneous introduction of a few drops of the patient's blood into a guinea-pig after about two weeks led to active phagocytosis on the part of approximately two-thirds of all the mononuclear cells. The same writer has described auto-phagocytosis in the blood of tertian malaria and chronic lymphatic leukemia (3). More recently Blumenthal (3a) has observed the phagocytosis of both erythroblasts and erythrocytes on the part of non-granular, large mononuclear leucocytes in the bone marrow of adult and embryonic rabbits.

In pleuritic exudates I have repeatedly observed the same phenomenon and was struck as in the other instances with the apparently purposeless character of the process. The assumption of the presence of auto-hemopsonins does not help us in our understanding of the process, and bearing in mind the absence of phagocytosis in other cases in which active blood destruction is taking place, the formation of auto-antibodies in these exceptional cases can hardly be viewed as anything more than a freak response, and hardly as a protective reaction. I do not wish to convey the idea with this statement that I regard the leucocytes as non-essential in the defensive reaction of the body against its own cells and cell degeneration products. I am merely of the opinion that physical phagocytosis on the part of the leucocytes does not play an essential rôle during this process, while I would not deny their chemical coöperation by any means, even though there is no direct proof of such action at the present time. Extravascular phagocytosis, that is phagocytosis occurring outside of the blood vessels, within the tissues, I would view in the same light, viz., as accidental and purposeless. This suggests itself at once in connection with the phagocytosis of leucocytes, young connective tissue cells and parenchyma cells on the part of cancer cells. If such pictures are to be construed as evidence of a defensive reaction at all it must be admitted that this reaction must be in the nature of a defensive reaction on the part of the offending cell and not of the body which is the seat of the disease. In my own studies I have never seen any evidence to suggest that phagocytosis of cancer cells by the leucocytes or other cells of the body plays any rôle of importance, and I think we may rightfully conclude that physical phagocytosis is a negligible quantity so far as the subject under discussion is concerned.

As regards the second method of possible defence, namely, by the production of antibodies directed against the body's own cells and cell products, relatively little is as yet known. Some of the reported findings lack confirmation, and in some instances the conclusions are probably based upon a misinterpretation

of the observed facts. Referring to the question of the possible formation of autolysins, Ehrlich and Morgenroth (4), in fact, inclined to the idea that this does not take place (*horror autotoxicus*) at all, and that its occurrence would naturally lead to dire consequences for the entire organism. In their experiments in this direction they were able to bring about the formation of iso-hemolysins, but not of auto-hemolysins. While this may be so under relatively normal conditions there is some evidence to show that in the event of hemorrhages into the large cavities of the body auto-hemolysin formation may after all occur, and Michaelis (5) and Kober (6) interpret the hemoglobinuria which has been observed in such cases in this sense. Metalnikoff (7) obtained auto-spermatotoxin production in guinea-pigs which had been treated with their own spermatozoa. Lindemann (8), as the result of his studies of toxic nephritis, comes to the conclusion that the nephritic process is due only indirectly to the primary toxic agent (chromium salts, cantharidin), but is directly the outcome of the formation of auto-nephrotoxins, in consequence of the resorption of a primary focus of nephritis. Nefedieff (9), Bierry (10), Ascoli and Figari (11) came to similar conclusions. The former thus observed that the serum of rabbits in which one ureter had been ligated became nephrotoxic; Bierry noted the same after ligation of the renal artery, and Ascoli and Figari after a unilateral nephrectomy. Donati (12) explained the clinical symptoms and pathological findings following unilateral ligation of the blood-vessel of the kidney in rabbits upon the same basis.

While the reports of these observers sound quite convincing, it is to be noted that the findings of others have not been in accord, and I for one cannot help feeling that the term nephrotoxin is used too loosely and frequently without adequate experimental basis. It does not follow by any means that the toxic agent or agents with which these gentlemen have been dealing are true antibodies, in the sense of Ehrlich, and with these we are at present concerned. Pearce and Sawyer (13), while confirming the results of Lindemann and Bierry, viz., that sera of dogs having nephritis are toxic for normal kidneys, likewise feel that the phenomenon should not be used as a basis for the explanation of the pathology of nephritis in general.

Equally indefinite is our knowledge of the formation of auto-antibodies in pregnancy. Weichardt (14) more especially attributes the occurrence of eclampsia to the formation of auto-syncytiolysins and their effect upon syncytial cells which have entered the general circulation, an endotoxemia, so to speak, resulting. However this may be, so much seems to be fairly well established, through the researches of Liepmann (15), that in pregnancy placental constituents are actually demonstrable in the blood serum.

While the preceding writers have directed their attention to the existence of cytotoxins, Centanni (16) believes he has demonstrated the existence of auto-precipitins, referable to auto-immunization with cell products. He states that such substances which he terms auto-cytoprecipitins, are frequently encountered in the sera of animals and of man in various diseases and intoxications. He remarks that such sera give rise to a precipitate already on simple dilution with normal salt solution and that they are not specific. He correlates auto-precipitation with fibrin formation on the one hand and hetero-precipitation on the other, viewing fibrin formation as a reaction of the body to the products

of the physiological metabolism of its own normal organs, the auto-cytoprecipitins as the reaction to the absorption of products of its own diseased organs, and the hetero-precipitins as reaction to foreign principles. Of special interest is his observation that the serum of sheep affected with hepatic distomatosis contains precipitins for extracts of normal liver, after partial autolysis has taken place in the latter. Such sera are capable of binding complement *per se*, but the fixation is more intense in the simultaneous presence of liver extract (17).

Sabrazès and Muratet (18) speak of the occurrence of auto-agglutination of the red cells in trypanosome infections, but have made no analytical study of the reaction. Widal, Abrami and Brule (19) report that they have observed marked auto-hemagglutination in four cases of hemolytic acholuric (hematogenic?) icterus, and these authors, as well as Worobjeff, explain the symptom complex upon the basis of a hemolytic auto-intoxication, dependent upon a primary "blood disease."

Lüdke (20) refers the anemia of chlorosis to the action autolysins derived from the developing ovaries, supposing that the red cells carry coreceptors for these bodies, but adduces no experimental evidence.

Of interest is the observation of Friedemann (21) and Wohlgemuth (22), who found that the pancreatic juice contains an auto-hemolysin which apparently has the structure of an amboceptor and is activated by means of lethicin. It is thus closely related to the snake venom and bee poison lethicids,<sup>2</sup> and can accordingly not be viewed as an antibody. It is quite conceivable that under pathological conditions an auto-hemolytic effect might be produced by such a product and that the serum of the patients might possess hemolytic properties, which accordingly would be erroneously attributed to an antibody. As I shall show later on, it is important to bear the possible formation of hemolysins of this order in mind in connection with cell degeneration in general. In this connection it is interesting to note that v. Bergmann was able to protect animals against the fatal consequences of pancreatic auto-digestion by previous immunization with pancreatic material. Such protection might naturally be referred to a corresponding antibody formation and it would suggest itself that the same probably would be attempted by the diseased animal itself. The proof, however, is lacking.

While the above observers essentially deal with more or less hypothetical auto-antibodies which are comparable to the antibodies which result on immunization with foreign cells and cell products, there is a tendency at present to view the complement fixation observed in syphilis as referable to the formation of auto-antibodies of a different type. The nature of the original technique, to be sure, would suggest that they represent amboceptors, and might hence after all be comparable to or identical with cytotoxins in the sense of Ehrlich. Satisfactory proof that such is actually the case has, however, not been afforded. All the observed phenomena connected with the process of complement fixation seem quite readily explained on the basis of physical adsorption, and there is nothing to render necessary the assumption of an amboceptor nature on the part

<sup>2</sup> The term lethicid is here used in the sense of Kyes, but may be inapplicable, as suggested by Bang (*Biochem. Zeit.*, 1908, 11, 321).

of the reacting serum component. Accordingly I think it is well, for the present at least, to regard them as reaction products of a separate order. They may indeed be auto-antibodies, but they are not antibodies directed against the specific parasite of lues. If this view be correct (23), in the case of syphilis then it would probably also be permissible to speak of auto-antibody formation in other pathological conditions in which complement fixation has been observed in the presence of various organ extracts as antigens. Centanni's findings in sheep distomatosis would thus fall under this order. The same would hold good for frambesia, certain trypanosome infections (24) and cancer.

I have pointed out in a previous paper (25) that complement fixation is frequently observed in malignant disease, using cancer extract as antigen, and I expressed the hope at the time that the technique could be so perfected that the method could be used for diagnostic purposes. This hope has not been fulfilled. We have not been able to demonstrate fixation in more than some sixty per cent. of the cases and we have found that syphilitic cases react with cancer antigen and *vice versa*. It is true that the fixation in cases of cancer is usually only of moderate intensity and often only slight, while the syphilitic reaction is more often absolute; but in syphilis also slight reactions are encountered and in cancer they may be likewise intense. Our observations in this direction have been fully confirmed by others, whose work in part at least has been synchronous with our own (26). While the diagnostic significance of complement fixation in cancer is thus only relative, the principle involved is important from the standpoint of our present paper. If the reaction in syphilis is due to the formation of auto-antibodies, the same undoubtedly holds good for cancer; the differences which exist are probably not qualitative, but purely quantitative.

In this connection I would refer to the observation that inactivated sera may under various pathological conditions bind complement *per se*, viz., in the absence of any special "antigen." The phenomenon was first observed by Neisser and Doering (27) in a case of impending uremia, and has since been studied by Neisser and Friedemann (28), Laqueur (29), Hedinger (30), Wolze (31), Senator (32), Lüdke (33), Noguchi (33a), v. Bergmann and Keuthe (34), who noted it in isolated cases of pneumonia, sepsis, carcinoma, leukemia, uremia, etc. We have notably met with this reaction in hepatic cirrhosis, and v. Bergmann and Salvini (35) were able to produce it artificially in phosphorous poisoning, and Eva Hoffmann (36) in uremia and nephritis. More recently Fiessinger (37) reports that he could demonstrate antibodies of this order in the serum of patients with severe degenerative lesions in the liver, such as hepatic cirrhosis, with the complement fixation method, and he views these, in our estimation without proper basis, as hepatotoxins. I am strongly tempted to share v. Bergmann's view that the reaction in question is due to an interaction between relatively large quantities of circulating degenerative products of body cells and corresponding auto-antibodies with consequent adsorption of complement.

In conclusion there remains for consideration the possibility of an auto-antiferment formation. Generally speaking this might be anticipated whenever cell destruction is going on, since the intracellular ferments must then of necessity be liberated. But unfortunately our knowledge of these ferments is still quite meager and the technique involved in their demonstration sufficiently

complicated as to render the study of their reaction products, the antiferments, correspondingly difficult. A more detailed investigation of auto-antiferment action has indeed been made only in the case of antitrypsin.

Fermi and Pernossi (38), Camus and Gley (39), Pugliese and Coggi (40), as well as Hahn (41), have demonstrated that normal blood serum in itself possesses antitryptic power. Other observers, such as Ascoli and Bezzola (42), Kolaczek (43), Bittorf (44), and Wiens (45), then showed that under pathological conditions this could be either increased or diminished, and since Achalmé (46) had succeeded in immunizing animals against trypsin, the conclusion suggested itself that the increased antitryptic action in disease might well be due to "antitrypsin." Ascoli and Bezzola's work had reference essentially to pneumonia, where they found a marked increase up to the time of the crisis, while afterwards coincidently with the disappearance of the local signs there was a considerable decrease. Kolaczek, Bittorf and Wiens noted deviations from the normal in various infectious diseases. More systematic observations have recently been recorded by Brieger and Trebing (47). These investigators were led to a more detailed study of the phenomenon in question by the fact that they found an increased antitryptic content of the serum in a case of cancer, which remained constant during a period of observation extending over several weeks. They then found that an increase of antitrypsin occurs in a large percentage of cancer cases, namely in over 90 per cent., and that this increase may under certain conditions be of diagnostic importance. v. Bergmann and Meyer (48) confirmed this work with a different method, but found, like Brieger and Trebing, that a similar increase may be observed in some 20 per cent. of non-cancerous conditions, such as pernicious anemia, Basedow's disease, severe nephritis, sepsis, tabes, paresis, etc. It is to be noted, however, that the increase which is observed in these diseases is by no means constant, and that with the possible exception of pernicious anemia and Basedow's disease, such an increase is more frequently absent than present. Dr. Mary Roche (49) has recently collected the cases which were examined in my laboratory during the fall months and has arrived at similar conclusions.

The above survey will furnish an idea of the most notable work which has thus far been accomplished bearing on the problem of auto-antibody formation. My own impression in going over the work has been that in many instances the question may rightfully be asked, whether the results obtained really prove the point at issue, whether the reactions which the various observers have noted are really referable to antibodies. The whole question seems to hinge on the definition of the word antibody. In our usual parlance we mean thereby certain reaction products which are formed by the animal body as a result of immunization with foreign cells or cell products. These reaction products may be of the nature of antitoxins, of agglutinins, of cytotoxins, of precipitins and of anti-

ferments. As we have no proof whatever that the substance or substances which give rise to the so-called Wassermann reaction in syphilis, and the corresponding reaction in cancer, are antibodies of this order, we are manifestly not justified in classifying these bodies as antibodies in the original sense of the word. So far as the other anti-reactions are concerned it is manifest that they would more readily fall under the category of antibodies, if we can properly extend the definition of the term so as to include reaction products resulting upon immunization with the body's own cells or cell products. This seems in a measure to be justifiable in view of what we know of the formation of iso-antibodies and some of the experiments recorded above. In others, the proof has to my mind not been furnished, and I feel more particularly in connection with the question of auto-cytotoxin production that much more additional evidence is necessary before this can be regarded as established. If once established, however, we should be forced to the anomalous conclusion that whereas cytotoxin production as a result of immunization with foreign cells can be viewed as a defensive reaction, auto-cytotoxin production against the body's own cells would be in the nature of an offensive reaction, a link in a vicious circle. This would certainly follow, unless the action of the auto-cytotoxin production were directed against diseased or otherwise anomalous cells, and not against normal cells of the same type; but this view in turn would presuppose the existence of specific receptors on the part of the diseased or anomalous cells, which are not possessed by the normal cells. Of this we have no evidence, and it has been noted more specifically in the nephrotoxic experiments referred to above that *normal* cells were attacked by the supposed autotoxin. As I have said, it seems to me that this question cannot be regarded as settled, and that the experimental evidence is thus far insufficient to warrant the recognition of auto-antibodies of this order.

I have reviewed this subject as outlined in order to show from what sides the study of the body's own defense against malignant disease may be attacked with the likelihood of making any definite progress. The only points along these lines that are definitely tangible, and a further study of which may lead to fruitful results, are the increased antitryptic content of the blood serum and the

demonstration that in malignant disease, as in syphilis, substances appear in the blood which are characterized by their increased affinity for complement in the presence of certain organ extract constituents. Research along these lines is still in progress. In the present paper, however, we wish to draw attention more particularly to another line of research, looking into the body's defences against its own cell constituents, which has taken for its point of departure the well known tendency on the part of malignant disease to lead to anemia. Clinical as well as experimental evidence goes to show that this tendency is largely independent of coincidental bacterial infection and referable to the growing tumor itself.

Panzacchi (50), Micheli and Donati (51), and Kullmann (52) thus demonstrated that tumor extracts contain hemolytic substances, but to Weil (53) belongs the credit for having shown that necrotic tumors contain hemolysins which differ markedly from those that can be extracted from non-necrotic tumors. The latter apparently belong to the same class of hemolytic substances which can be extracted from normal organs (liver, kidney, pancreas) and must be viewed as complex hemolysins, requiring an additional factor for their activation which Weil found present in extracts of normal red cells. The hemolysins in necrotic tumors, on the other hand, are apparently simple hemolysins requiring no additional "complement" for their activation. Weil has suggested that these latter hemolysins enter the blood and contribute to the anemia and the cachexia of malignant tumors. In a series of studies (54) the same investigator has actually shown that hemolysins are frequently present in the blood serum of malignant disease, which can be demonstrated by allowing the cancer serum to act upon the red corpuscles of non-cancerous patients. Very curiously, the patient's own corpuscles, *in vitro* at least, show an increased resistance to their own hemolytic serum. Crile (55) has come to the same conclusions which Weil originally reached in his lympho-sarcomatous dogs, and seems to attach considerable importance to the hemolytic action of cancer serum, from the diagnostic standpoint, although he finds the same reaction in cases of tuberculosis.

The work of these two investigators represents the starting point of our own studies. It seems clear from what has been said that hemolysins are formed in malignant tumors and that these substances enter the circulation. There seems to be good evidence of their toxic nature and the question hence suggests itself: in what manner does the animal body attempt its own defence against such substances? In considering the possible lines of investigation in this direction the thought naturally suggested itself that even under normal conditions a mechanism might be active which would antagonize hemolytic agents, analogous to the normal inhibitory action

which normal blood serum possesses in reference to certain ferments. It was hence decided to investigate this question, first in reference to some non-specific, general, hemolyzing agents, and to extend the examinations, if evidence of a normal inhibitory mechanism were found, so as to include hemolyzing agents of various kinds, and among them the tissue and serum hemolysins of cancer, syphilis and tuberculosis. The first portion of the work is embodied in the present report.

The general plan of work will appear from a survey of our initial studies with saponin.

#### SAPONIN EXPERIMENTS.

Serum and corpuscles were examined separately. In the case of the latter, varying amounts of saponin were incubated for 30 minutes with constant quantities of the washed human corpuscles, and the results then read. With the serum, varying amounts of saponin were incubated for 30 minutes with constant quantities of serum, after which constant quantities of corpuscles were added. For convenience sake, washed chicken corpuscles were uniformly used to this end. Any deviations from the normal could thus be attributed directly to the human corpuscles, on the one hand, and to the patient's serum on the other. The corpuscles, both human and those of the chicken, were washed three times and then made up into 5 per cent. emulsions (corresponding to the full blood, or as 2.5 per cent. emulsions, corresponding to the isolated red cells) in 0.85 per cent. saline. The serum was diluted in the proportion of 1 in 10 with saline.

Two strengths of saponin were employed. The one solution, *A*, represents a 0.1 per cent. solution in 0.85 per cent. saline, while the second, *B*, represents a 1 in 10 dilution of *A*. Series of tubes were charged with varying amounts of these solutions and the volume was always brought to 1 c.c. with saline, after which the human corpuscles were added in the one series, and the diluted human sera in the other, 0.5 c.c. of either being the constant amount of these, as also of the chicken corpuscles, as described above. Preliminary experiments with normal human corpuscles showed that 0.1 c.c. of the *B* solution (*B*-1) of saponin will rarely hemolyze 0.5 c.c. of normal corpuscles; as a rule, there is not a trace of hemolysis with this concentration. In many normal instances the same is seen in tube *B*-2; but in others there is more or less extensive hemolysis. Beyond this, namely, in *B*-3, and *B*-4, etc., there is complete hemolysis.

With normal sera no hemolysis is usually obtained in either *B*-9 or in *A*-1;

occasionally *A-1* shows more or less extensive hemolysis, but this is the exception. Beyond *A-1* there is complete hemolysis. Hence for routine work it is necessary only to put up tubes *B-1* to *B-4* for the corpuscles and *B-9* to *A-4* for the serum.

An experiment of this order is recorded below. It is to be noted that in this particular instance the corpuscle experiment in contradistinction to the serum experiment, has reference not to human red cells, but to those of the chicken. This will show very clearly the marked protective power on the part of the blood serum, as the range between corpuscle hemolysis and serum-corpuscle hemolysis is even wider than in the case of the human red cells.

	I Serum.	II. Corpuscles.
<i>A-3</i>	Hemolysis	Hemolysis
<i>A-2</i>	Hemolysis	Hemolysis
<i>A-1</i>	Inhibition	Hemolysis
<i>B-9</i>	Inhibition	Hemolysis
<i>B-8</i>	Inhibition	Hemolysis
<i>B-7</i>	Inhibition	Hemolysis
<i>B-6</i>	Inhibition	Hemolysis
<i>B-5</i>	Inhibition	Hemolysis
<i>B-4</i>	Inhibition	Hemolysis
<i>B-3</i>	Inhibition	Hemolysis
<i>B-2</i>	Inhibition	Hemolysis
<i>B-1</i>	Inhibition	Hemolysis
<i>C-9</i>	Inhibition	No hemolysis
<i>C-8</i>	Inhibition	No hemolysis

Up to the present time we have examined the sera and corpuscles of some 200 cases, including a number of supposedly normal individuals. The list covers a fairly wide range of pathological conditions, as is apparent from the accompanying tables, in which the cases are arranged in four groups. Group I includes those cases which showed no hemolysis in either *B-9* or *A-1* (corresponding to 0.00009 and 0.0001 gm. of saponin respectively). Group II covers those which showed no hemolysis in *B-9*, but a variable degree of hemolysis in *A-1*. In Group III those cases are collected in which hemolysis occurred in both *A-1* and *B-9*. Group IV finally represents a small number of cases in which no hemolysis occurred in either *B-9*, *A-1*, *A-2*, or still stronger solutions of saponin.

We interpret our findings by inferring in Group I that the sera of the cases collected under this heading have the same antihemolytic power as is observed under strictly normal conditions, while in Group II this is less; in Group III, still less; and in Group IV, increased. Most of the normal cases fell under Group I, while a smaller percentage is to be found in Group II. We have never seen a single instance where a normal individual showed a decrease in the antihemolytic power of such extent as to fall under Group III. We have similarly never observed a material increase extending beyond A-1; occasionally A-2 is not completely hemolyzed, but a more marked inhibition does not occur.

The following Table I gives a summary of our cases arranged under the heading of the different diseases; the results show the number of cases examined and the percentage values for hemolysis at various concentrations of the saponin solution.

TABLE I.

	Total no of cases	I. Hemolysis in B-9 and upward.		II Inhibit. in B-9 only.		III Inhibit. in B-9 and A-1		IV. Inhibit. beyond A-1	
		No of cases	Per cent.	No of cases	Per cent.	No. of cases.	Per cent	No. of cases.	Per cent.
Normal cases.....	24	0	0	7	29	17	71		
Malignant tumors.....	15	3	20	6	40	6	40		
Non-malignant tumors.....	5	1	20	2	40	2	40		
Appendicitis.....	1	—				1	100		
Pernicious anemia.....	1	—				1	100		
Syphilis.....	15	12	80	2	12	1	8		
Tabes.....	2	2	100						
Syphilitic mental cases.....	3	3	100			0			
Non-syphilitic mental cases.....	10					10	100		
Tuberculosis.....	90	51	56	19	21	20	23		
Chancroid.....	1							1	100
Multiple sclerosis.....	1					1	100	1	100
Apoplexy.....	1								
Ulcer of the stomach.....	4			2	50	2	50		100
Arteriosclerosis.....	1								
Neuroses.....	1			1	100				
Gonococcus infections.....	3	1	33	2	66				
Chronic arthritis.....	1			1	100				
Staphylococcus infections.....	1			1	100				
Streptococcus infections.....	1			1	100				
Pernicious vomiting of pregnancy.....	1			1	100				
Cholelithiasis.....	4							4	100
Strongyloides infection.....	1					1	100		
Carbon monoxide poisoning.....	1	1	100						

The behavior of the pathological sera was most interesting. As a glance at the table above will show, a deviation from the normal was observed in only three of the cancer cases, and in one of these a history of syphilis could not be excluded. This means, of course, that so far as saponin is concerned the antihemolytic power of the blood serum is neither materially increased nor diminished. It might be argued that this observation has no direct bearing upon the question of the antihemolytic protective mechanism in malignant disease, and that in cancer a more specific protective mechanism may be brought into play, which would be tuned to specific cancer hemolysins. This is, of course, quite conceivable and the problem was accordingly put to the direct test as will be discussed more fully later on.

Quite interesting further is the observation that in pernicious anemia no evidence of either an increased or a diminished antihemolytic power was obtained, which also could be interpreted as merely showing that no change in the antihemolytic action exists so far as saponin is concerned. We will have occasion to revert to this question on a future occasion.

The most remarkable result in this series of observations was obtained in cases of syphilis and tuberculosis. As a glance at Column I will show, hemolysis in B-9 was obtained with few exceptions in these two diseases only. In interpreting the result, two possibilities suggested themselves: on the one hand, it was possible that the increased tendency to hemolysis by saponin was due to a diminution in the amount of antihemolytic substance, and on the other, that the diminished antihemolytic effect was in reality due, not to a negative, but to a positive factor, that is, to the presence of hemolysins in the blood serum of the patient, the effect of which would be obtained either independently or superadded to the action of the saponin. The second possibility suggests itself directly since Crile (56) reports that the blood serum of tuberculosis may be hemolytic for normal corpuscles, while Peskind (57) has demonstrated the same for syphilis. We found, however, that this explanation is not applicable in the present instance. We could demonstrate by suitable controls that with rare exceptions and with the degree of dilution which we employed tubercular sera do not

possess any hemolytic action whatever upon chicken corpuscles. Very rarely only does one see a very faint pink in these controls, and it is noteworthy that this occurred at times even though the corresponding *B-9* saponin tube showed no trace whatever of hemolysis. Our results in this respect are so striking that we have not the least hesitancy in affirming that the marked hemolysis in the *B-9* tubes cannot possibly be due to the action of a tubercular hemolysin, acting either independently or in addition to the saponin effect. We believe that the conclusion is warrantable that the increased hemolytic effect with saponin, which is so frequently observed in tuberculosis, must be referable to a decrease in the anti-hemolytic components of the blood serum. We have reason to think that the same holds good for syphilis.

While any inferences that may be drawn from these experiments apply for the present at least to the mechanism of saponin hemolysis only, the results seem to be important in principle. They show quite clearly that a certain type of anemia may be referable primarily to a diminution in the quantity of a normal protective agent, and only indirectly to the effect of the hemolysin upon the corpuscles. This protective power is indeed very considerable, if we bear in mind that our results were obtained with sera which had been diluted tenfold at the start, and further diluted by the addition of double the volume of the saponin solution. It is true that our sera were usually tested only against a 5 per cent. emulsion of corpuscles, but we could show that its protective action, even in the dilution which we employed, was in reality of a much wider scope.

#### CHARACTERISTICS OF THE ANTIHEMOLYTIC SUBSTANCE.

Regarding the nature of the antihemolytic substance, the following data were obtained.

Heating experiments showed that exposure of normal human serum for 30 minutes at 52° C. does not interfere with its normal antihemolytic action so far as saponin hemolysis goes.

The following experiments were undertaken to ascertain the part which the proteins of the serum take in the antihemolytic action of the blood serum. The material used was the pleural exudate from a tubercular patient (Sch.) which in its original condition gave absolute inhibition in *B-9* and almost complete hemolysis in *A-1*.

50 c.c. of this serum after careful centrifugalization, were treated with 50 c.c. of a neutral saturated solution of ammonium sulphate and the precipitated globulins were filtered off the next day. The filtrate, when brought to the proper dilution and sodium chloride concentration showed no manifest antihemolytic effect whatever, from *A-5* to *B-7*. In *B-6* yet there was very extensive hemolysis and in *B-5* even there remained a large cup of undissolved red cells.

I am nevertheless inclined to think that underlying this apparent lack of antihemolysis there is still an antihemolytic effect, since control experiments with corresponding amounts of ammonium sulphate in saline, with even lower amounts of saponin, showed instantaneous hemolysis, whereas the hemolytic action in the presence of the filtrate took place gradually on incubation. F. Sachs (58) has recently observed that soap hemolysis will occur instantaneously in the presence of OH ions, but he remarks that with saponin the phenomenon was only suggestive and then only when sodium hydrate had been used. Our experience with ammonium sulphate shows that it occurs instantaneously, while the same salt without the saponin causes no hemolysis whatever during the time limit (30 minutes) that we allowed our preparations to remain in the incubator. Bearing this effect in mind, our conclusion, that the filtrate from which the globulins have been removed, has still a certain degree of antihemolytic action, does not seem unwarrantable. However, we have not followed up this point any further.

The globulin fraction was next tested, after bringing it to the proper degree of dilution and sodium chloride concentration. It was found that with *B-9* and *A-1* there was complete inhibition, partial inhibition in *A-2* and complete hemolysis only in *A-3*. It would thus seem as though almost the entire antihemolytic effect were centered in the globulin precipitate. To ascertain to what extent the eu- and the pseudoglobulin fractions are concerned in the question, the entire globulin precipitate, dissolved in saline, was dialyzed against running water for twenty-four hours, and the two portions tested, after bringing them to the proper degree of dilution and sodium chloride concentration. It was found that the euglobulin fraction had lost much of the original antihemolytic action, as complete hemolysis was obtained not only in *A-1* and *B-9*, but extensive hemolysis was yet obtained with *B-8* and *B-7*; a complete

antihemolytic effect was not obtained until *B-5* was reached. Examination of the pseudoglobulin fraction showed a corresponding loss even exceeding that of the former; complete hemolysis was obtained as far down as *B-7*, and even in *B-5* it was extensive. The impression was thus gained that the antihemolytic substance had either disappeared or become inactive. That the time element plays no rôle at this point is clear from the fact that sera can be preserved much longer and still retain their antihemolytic action.

In order to ascertain whether the cholesterin of the blood serum might be responsible for the antihemolytic effect, one set of tubes was charged as usual with serum and saponin plus an extra volume of saline, while the second set received corresponding amounts of the same serum with saponin, and in place of the saline, one volume of an emulsion of cholesterin in saline, which had been centrifugalized as free from cholesterin in suspension as possible. The result is seen below:

	I. Serum—Saline—Saponin.	II. Serum—Cholesterin—Saponin
<i>B-9</i>	Inhibition (absolute).	Absolute inhibition.
<i>A-1</i>	Inhibition (absolute).	Absolute inhibition.
<i>A-2</i>	Almost complete hemolysis.	Absolute inhibition.
<i>A-3</i>	Almost complete hemolysis.	Partial hemolysis.

It is thus evident that cholesterin, even in minute doses, is capable of increasing the antihemolytic action of normal blood serum quite materially, and it is noteworthy that the same effect is obtained with cholesterin in the absence of serum, as is shown in the following experiment:

	I. Corpuscles—Saline—Saponin.	II Corpuscles—Cholesterin—Saponin.
<i>B-5</i>	Practically complete hemolysis.	Extensive inhibition.
<i>B-6</i>	Complete hemolysis.	Extensive inhibition.
<i>B-7</i>	Complete hemolysis.	Extensive inhibition.
<i>B-8</i>	Complete hemolysis.	Fair Inhibition.
<i>B-9</i>	Complete hemolysis.	Slight inhibition.
<i>A-1</i>	Complete hemolysis.	Hemolysis complete.
<i>A-2</i>	Complete hemolysis.	Hemolysis complete.

Our results in this respect are thus in perfect accord with those of Ransom (59), who, already in 1901, noted the antihemolytic action of cholesterin toward saponin, but he does not seem to have

utilized the principle involved in the study of the protective mechanism of the animal body in disease.

It would of course be tempting to follow up this line of investigation by corresponding studies on the cholesterol content of the blood serum in syphilis and tuberculosis. But for the present we were obliged to content ourselves with the mere recognition of the possibility that the decreased antihemolytic content of the blood serum in the two diseases mentioned may be due to a decrease in the cholesterol content, and that our saponin-hemolysis technique may constitute a convenient method for estimating that component in the blood serum. A direct investigation of this question is now in progress.

So far as our present experience goes it would seem that a diminution in the cholesterol content of the blood serum of tubercular patients is principally observed in actively progressing cases, while in mild cases and early lesions normal values are usually obtained. We could thus note that among sanatorium patients who were confined to bed a decrease in the antihemolytic power of the serum was observed in nearly every case, while among those who were up and about normal values were frequently found. Interesting further is the observation that the injection of tuberculin *per se* does not appear to affect the antihemolytic power of the serum at all. The actual significance of the phenomenon still remains to be elicited, and, for the present at least, we should like very much to reserve this problem for our own investigation.

In syphilis also we have found that a lowered antihemolytic action of the serum is essentially observed in active cases, in which a positive Wassermann reaction can be obtained. On this point also we wish to report in detail on some future occasion.

If now we turn our attention to the behavior of the corpuscles toward saponin, we find that, unprotected by serum, they are hemolyzed very readily even under normal conditions by much smaller amounts of the reagent. In 50 per cent. of the controls partial hemolysis, at least, occurred already in the B-2 tube, corresponding to 0.00002 gm. of saponin to 125,000,000 red cells. Even in the B-1 group there are a few normal individuals, while only 30 per cent. have corpuscles which resist the saponin of B-2

altogether. Beyond this we have never seen resistance in normal individuals.

So far as pathological conditions are concerned, we have found no constant deviations from the normal values in any one disease. No increased resistance toward saponin is noticeable in those two diseases, more especially where increased resistance has been observed toward the respective hemolytic sera, viz., in cancer and syphilis (Crile and Peskind). If there be any tendency at all toward a deviation from the normal, it is certainly in the direction of increased vulnerability, and not the reverse. This observation, as also the fact that the corpuscles in tubercular patients and of the single case of pernicious anemia (which was very severe) show no essential deviation from the normal, we would emphasize more particularly, as it seems quite important in principle.

#### TOLUOL HEMOLYSIS.

For workers with organ extracts it is important to bear in mind that toluol, which is so extensively used as a preservative, has very decided hemolytic properties. We found that the solubility of toluol in saline is such that 0.5 c.c. of the saturated solution will bring about the complete hemolysis of 0.5 c.c. of a 5 per cent. emulsion of chicken corpuscles within 30 minutes, at 37° C.

Agents in which toluol is more readily soluble, and which themselves are soluble in saline, bring about a corresponding increase in the degree of hemolysis. Alcohol acts in this manner. For this reason an alcoholic extract of an organ, containing toluol, is more strongly hemolytic than a corresponding saline extract. Working with both saline and alcoholic extracts, which *per se* were not hemolytic, but in which the presence of hemolysins was simulated by the toluol, we found that as in saponin hemolysin blood serum exercised a marked antihemolytic effect.

We have studied 55 sera from different diseases, and corpuscles from 25 cases, in their behavior toward toluol as an hemolytic agent, but have been unable to observe any differences worth noting, as compared with the normal.

So far as the nature of the antihemolytic component of the serum is concerned, we have ascertained that the inhibitory substance is

precipitated with the globulins on salting with ammonium sulphate, and that the inhibitory action of both the euglobulin and the pseudoglobulin fraction is practically alike. Cholesterin plays no rôle in the process.

#### SOAP HEMOLYSIS.

Soap was chosen as the next hemolytic agent, because its universal presence in the fluids and tissues of the body renders it especially important as a possible factor in the production of anemia. Through the researches of Noguchi (60) and v. Liebermann (61), working independently of one another, it was first shown that the blood serum is capable of inhibiting the hemolytic action of soaps, and that their innocuous existence in the blood in concentrations which *in vitro* would be sufficient to cause hemolysis, is no doubt dependent upon the antihemolytic action of the blood serum. As it is *a priori* not impossible that this antihemolytic mechanism may in disease be more or less seriously impeded, it would follow that under pathological conditions soap hemolysis might actually take place in the body, and that by suitable experiments it should be possible to demonstrate deviations in the antihemolytic action of the serum. To this end we have investigated the effect of the serum upon soap hemolysis in a fairly large number of the more important diseases, directing our attention more particularly to cancer, tuberculosis and syphilis.

As in our saponin experiments, we first ascertained the concentration of the soap solution in which complete hemolysis of a constant quantity of washed red corpuscles will occur. Subsequently it was determined to what extent the concentration of the soap solution must be increased in order to bring about hemolysis of the same quantity of red cells in the presence of constant quantities of normal blood serum. After this, pathological sera were similarly examined.

For routine work we employed a 0.5 per cent. solution of sodium oleate in 0.85 per cent. saline solution. The sera were diluted 1 : 10, and as in our saponin work we used a 5 per cent. emulsion of thrice-washed chicken corpuscles. Our soap was the neutral sodium oleate marketed by Merck. The general arrangement of the experiments, the hemolytic power of the soap and the antihemolytic effect of normal blood serum is shown in the following scheme:

*I. Corpuscle Hemolysis.*

Soap solution.	Saline.	Corpuscles.	Result after 30 minutes incubation.
0.1 c.c.	0.9 c.c.	0.5 c.c.	No hemolysis.
0.2 c.c.	0.8 c.c.	0.5 c.c.	No hemolysis.
0.3 c.c.	0.7 c.c.	0.5 c.c.	Complete hemolysis.
0.4 c.c.	0.6 c.c.	0.5 c.c.	Complete hemolysis.
0.5 c.c.	0.5 c.c.	0.5 c.c.	Complete hemolysis.

*II. Serum Hemolysis.*

Soap solution.	Saline.	Serum.	Corpuscles.	Result after 30 further minutes of incubation.
0.1 c.c.	0.9 c.c.	0.5 c.c.	0.5	Inhibition.
0.2 c.c.	0.8 c.c.	0.5 c.c.	0.5	Inhibition.
0.3 c.c.	0.7 c.c.	0.5 c.c.	0.5	Inhibition.
0.4 c.c.	0.6 c.c.	0.5 c.c.	0.5	Inhibition.
0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5	Marked hemolysis.
0.6 c.c.	0.4 c.c.	0.5 c.c.	0.5	Complete hemolysis.
0.7 c.c.	0.3 c.c.	0.5 c.c.	0.5	Complete hemolysis.

In this connection it may not be out of place to refer to an observation which we made in the course of our experiments, which caused us a great deal of annoyance for a while. We obtained a neutral sodium oleate preparation from a local dealer, which at first showed perfectly normal behavior, hemolyzing quite readily and being inhibited by the addition of serum. After a few weeks, however, it lost its hemolytic power entirely, so that the substance became perfectly useless for hemolytic experiments. Chemical investigation showed that our neutral oleate had been transformed almost entirely into the oxy-salt. This possibility will have to be considered in the future in experiments in which an inhibitory effect is noted.

Regarding the manner in which sodium oleate brings about hemolysis; and in which blood serum inhibits this action, we would offer the following tentative explanation. While concentrated solutions of the oleate are actually neutral so far as phenolphthalein is concerned, solutions of such strength as are generally used in hemolytic work present a markedly alkaline reaction. This change can be readily observed on diluting a concentrated solution to which a drop or two of phenolphthalein has been added and in which no color can be discerned, when from a certain point off the red color of the sodium salt of phenolphthalein develops. On dilution of the soap we thus observe a hydrolytic dissociation leading to the liberation

of oleic acid on the one hand and caustic alkali on the other. In view of the fact that oleic acid is hemolytic *per se* and that hydroxyl ions, as I have shown before, may act as a katalysing agent, it would not appear far-fetched to explain soap hemolysis in dilute solution upon this basis. Hemolysis, it is true, will also be brought about by concentrated solutions of soap, in which this dissociation has not occurred, but it seems to me that the two conditions are not directly comparable, and that the hemolytic effect of concentrated solutions may very well be due to absorption of water from the red corpuscles. If our view regarding soap hemolysis in dilute solutions should prove to be correct, it would suggest itself that the inhibitory action of the blood serum could very well be due to a neutralizing effect of the albumins acting as acids upon the liberated alkali. We offer this explanation merely as a tentative one at this time and expect to report upon the question in some detail in a separate paper.

Our findings, so far as the antihemolytic action of the blood serum in disease is concerned, are collected in the following Table II.

TABLE II.

*Showing the extent of the antihemolytic power of the blood serum under various pathological conditions. The figures have reference to the concentration of the soap solution at which the antihemolytic effect of the serum (diluted 1:10) is first overcome (see example above).*

	Total no. of cases.	Hemolysis begins at 0.4 c.c.		Hemolysis begins at 0.5 c.c.	
		No. of cases.	Per cent.	No. of cases.	Per cent.
Normal cases.....	19	7	37	12	63
Malignant disease.....	11	7	63	4	36
Tuberculosis.....	20	18	90	2	17
Syphilis.....	6	1	17	5	93
Septic infections.....	4	3	75	1	25
Cholelithiasis.....	4	2	50	2	50
Measles.....	1	1	100		
Bone injuries.....	4	3	75	1	25
Pneumonia.....	2	1	50	1	50
Cirrhosis of the liver.....	3	1	33	2	66
Strongyloides infection.....	1			1	100
Osteomyelitis.....	1			1	100
Bronchitis.....	1	1	100		
Neurasthenia.....	2	2	100		
Typhoid fever.....	1	1	100		
Gonorrheal infections.....	1	1	100		
Pyelitis (calculous).....	1	1	100		
Nephritis.....	1	1	100		
Sciatica.....	1	1	100		
Non-syphilitic mental cases.....	5			5	100

An analysis of this table shows that whereas the majority of normal sera are capable of counteracting the hemolytic action of the soap solution up to 0.5 cubic centimeters, there are some individuals in which the antihemolytic power even normally does not extend beyond 0.3; hemolysis in some of these may indeed be complete at 0.4.

So far as the behavior of the sera in disease is concerned, it would appear that there is a decrease of the antihemolytic action under the most divers pathological conditions. It would hardly seem justifiable, however, to conclude that the anemia which may be an accompanying factor in some of these conditions is due to spontaneous soap hemolysis taking place in the body, as the same decrease may be observed in conditions in which no anemia at all is noticeable. We rather incline to the belief that the relatively slight deviation from the average normal values may be due to slight variations in the anion and kation content of the blood serum, and hence constitute an expression of the alkalinity of the blood. It follows that in the diseases which have been studied there is no evidence of a special defensive reaction on the part of the body so far as hemolytic agents of this order are concerned.

While our table shows that there is a decrease in the antihemolytic power of the blood serum in various pathological conditions, it is evident also that this is inconstant and cannot be utilized for diagnostic purposes. It is noteworthy nevertheless that in the case of soap hemolysis also a marked decrease in the antihemolytic power of the serum is demonstrable in a large proportion (90 per cent.) of the tubercular cases, and it would seem of interest to follow this up in a further study. In syphilis, on the other hand, normal relations are encountered.

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**PROTEOLYTIC ENZYMES AND ANTI-ENZYMES OF  
NORMAL AND PATHOLOGICAL CEREBRO-  
SPINAL FLUIDS.**

**By A. R. DOCHEZ,**

# PROTEOLYTIC ENZYMES AND ANTI-ENZYMES OF NORMAL AND PATHOLOGICAL CEREBRO- SPINAL FLUIDS.<sup>1</sup>

By A. R. DOCHEZ.

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Study of the proteolytic enzymes contained in leucocytes, and of the enzymotic and anti-enzymotic content of inflammatory exudates has revealed facts that explain many of the phenomena observed in the resolution and absorption of inflammatory products.

The white cells of an inflammatory exudate, and doubtless of the blood as well, may be divided into two groups according to their enzymotic activity. One group, formed by the polymorphonuclear neutrophile leucocyte, furnishes an enzyme which exhibits its greatest activity in neutral or alkaline medium. Opie<sup>2</sup> has called this enzyme leucoprotease. It is probably closely related to the  $\alpha$ -protease isolated from the spleen of the ox by Hedin.<sup>3</sup> On the other hand, the mononuclear phagocytic cells of inflammatory exudates and similar cells in inflamed lymph glands contain an enzyme, the lymphoprotease of Opie, which digests only in the presence of a weakly acid reaction, and is almost entirely inactive in neutral and alkaline media.

In most of the inflammatory conditions in the body the preponderant type of cell is the polymorphonuclear leucocyte. The exudates in these inflammations might be expected to exhibit proteolytic activity. Opie has shown that they do not always cause proteolysis because the blood serum, which is poured out with the leucocyte during the inflammatory reaction, contains an anti-enzyme. This anti-enzymotic action is able to check any enzyme that may be set free; thus the ability of any exudate to digest protein is de-

<sup>1</sup> Received for publication June 26, 1909.

<sup>2</sup> Opie, *Jour. of Exper. Med.*, 1905, vii, 316.

<sup>3</sup> Hedin, *Jour. of Physiol.*, 1904, xxx, 155

pendent upon the relative amounts of enzyme and anti-enzyme present. In sterile abscesses, produced by such a powerful inflammatory irritant as turpentine, the stimulus bringing about an accumulation of leucocytes is so great that in a short time enough cells and free enzyme are present to overcome completely the anti-enzymotic activity of the limited amount of blood serum that infiltrates the inflamed tissue. This preponderance of enzyme over anti-enzyme is facilitated because the pressure within the confined space, in which the exudate is contained, prevents such accumulations of fluid as occur in large body spaces like the pleural cavity. Consequently one would expect the contents of abscess cavities to be powerfully proteolytic. This proteolysis, which can be convincingly demonstrated *in vitro*, explains the rapid and often extensive solution of tissue that accompanies abscess formation.<sup>4</sup> It is not improbable that a purulent exudate, in which enzyme is more powerful than anti-enzyme, itself acts as an inflammatory irritant, and intensifies the exudation of serum and emigration of leucocytes.

Studies of Delezenne and Pozerski<sup>5</sup> and of Hedin<sup>6</sup> have shown that the blood serum contains both enzyme and anti-enzyme. The latter found that the globulin fraction exhibits the power of active proteolysis, whereas the albumin fraction manifests the characteristic anti-enzymotic action of the whole serum.

Little attention has been given to the proteolytic enzymes and anti-enzymes of the cerebro-spinal fluid. Edw. Müller<sup>7</sup> has noted the absence of anti-enzymes in normal spinal fluid. He also has compared the action of centrifugalized sediment of tuberculous and epidemic meningitis. The former he finds exhibits no enzymotic activity; the latter causes solution, when tested on Loeffler's serum plates. Chariolanza,<sup>8</sup> using spinal fluid, observed some slight inhibition of the digestive action of pus on serum plates. Whether the fluid was normal or not is not mentioned. In the present study, the absence of free proteolytic enzyme in normal and in most cases in pathological spinal fluids has been repeatedly observed.

<sup>4</sup> Opie, *Jour. of Exper. Med.*, 1906, viii, 536.

<sup>5</sup> *Compt. rend. Soc. de biol.*, 1903, lv, 327, 690, 693.

<sup>6</sup> *Jour. of Physiol.*, 1904, xxx, 195.

<sup>7</sup> *Münchener med. Woch.*, 1907, i, 354.

<sup>8</sup> *Medizinisch-Naturwiss. Archiv*, 1908, ii, 43.

The reaction of normal spinal fluid is faintly alkaline.<sup>9</sup> The low specific gravity<sup>10</sup> is probably attributable to the small protein content namely, from .02 to .04 per cent.<sup>11</sup> The protein of the normal fluid consists entirely of serum globulin, according to most observers, albumin being present only in pathological specimens.<sup>12</sup>

In view of the initial absence of both enzyme and anti-enzyme, the spinal fluid offers a favorable opportunity to determine what changes occur with pathological conditions. Since normal fluid contains no anti-enzyme, it is especially desirable to determine the properties of an inflammatory exudate containing polymorphonuclear leucocytes, and to know if reactions which produce such exudates are peculiarly severe in the spinal canal.

*Methods.*—The spinal fluids used for the following tests were found, upon examination, in some instances normal; and in others, pathological. The subsequent history of the cases confirmed the opinion formed from the examination of the specimen of fluid received. All the fluids were centrifugalized and serum was obtained free from cells. Only the supernatant fluid was used in the experiments, in order that it might be tested for the presence of proteolytic enzyme perhaps set free by disintegration of cells. The enzymotic activity of the fluid was determined by adding varying amounts to a flask containing beef serum, denaturalized by heat, as substrate. The whole volume was then made up to 25 c.c. by diluting with .85 per cent. salt solution, and the mixture allowed to digest for five days in the thermostat at 37° C. The coagulable protein was then precipitated by acidifying with dilute acetic acid, and boiling with an equal volume of twenty per cent. solution of magnesium sulphate. The coagulum was removed by filtering and the incoagulable nitrogen of the filtrate determined by the method of Kjeldahl. The amount of digestion is expressed in cubic centimeters of *N*/10 sulphuric acid. Controls were made in each case by boiling immediately equal quantities of the materials used, and determining the incoagulable nitrogen of the filtrate in the manner described above. The anti-enzymotic activity of the fluids to be

<sup>9</sup> Thomson, Hill and Halliburton, *Proc. of the Royal Soc.*, 1899, lxiv, 343.

<sup>10</sup> T. Sollmann, *Jour. of the American Med. Assn.*, 1903, xl, 1569.

<sup>11</sup> Pfaundler, *Jahrb. f. Kinderh.*, 1899, xlix, 264.

<sup>12</sup> Thomson, Hill and Halliburton, *loc. cit.*; T. Sollmann, *loc. cit.*

tested was measured by adding to each digestion flask, in addition to the cerebro-spinal fluid, a weighed amount of leucocytic enzyme. The mixture was placed in the thermostat for five days and the amount of digestion determined by the method given above. The degree of inhibition is estimated by subtracting from the figure representing digestion caused by enzyme alone that representing digestion by the same quantity of enzyme in the presence of measured amounts of spinal fluid.

#### ENZYME AND ANTI-ENZYME IN NORMAL SPINAL FLUID.

In the following experiments the fluid employed contained no microorganisms, and the subsequent history of the patients gave no evidence of any pathological condition of the meninges or central nervous system.

CASE I.—The patient from whom this specimen of fluid was withdrawn was admitted to the Presbyterian Hospital suffering with sero-fibrinous pleurisy. The spinal fluid was clear and colorless and contained no blood or sediment. The bacteriological examination was negative. The volume was 50 c.c.

#### *Enzymotic Activity.*

	Control.	After 5 days at 37°	Digestion
0.5 c.c. spinal fluid + substrate.....	1.05 c.c.	1.3 c.c.	0.25 c.c.
1.0 c.c. spinal fluid + substrate.....	1.2 c.c.	1.3 c.c.	0.1 c.c.
2.5 c.c. spinal fluid + substrate.....	1.6 c.c.	1.4 c.c.	None

#### *Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition
20 mg. leucoprotease + substrate ...	1.5 c.c.	16.9 c.c.	15.4 c.c.	
20 mg. leucoprotease + substrate				
+ 0.5 c.c. sp. fl. ....	1.65 c.c.	17.2 c.c.	15.55 c.c.	None
20 mg. leucoprotease + substrate				
+ 1.0 c.c. sp. fl. ....	1.8 c.c.	17.0 c.c.	15.2 c.c.	0.2 c.c.
20 mg. leucoprotease + substrate				
+ 2.5 c.c. sp. fl. ....	2.2 c.c.	17.25 c.c.	15.05 c.c.	0.35 c.c.

The slight differences seen here between free digestions and controls are within the limits of experimental error and cannot be considered as evidence of enzymotic or anti-enzymotic activity.

CASE II.—Spinal fluid was obtained from a patient in the third week of an attack of typhoid fever. It was normal in appearance and contained no blood or sediment. The bacteriological examination was negative.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
2.5 c.c. sp. fl. + substrate + 0.2 % acetic.....	2.8 c.c.	2.55 c.c.	None
2.5 c.c. sp. fl. + substrate (neutral) .....	2.8 c.c.	2.15 c.c.	None
2.5 c.c. sp. fl. + substrate + 0.2 % sod. carb..	2.8 c.c.	2.6 c.c.	None

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion	Inhibition.
20 mg. leucoprotease + substrate .....	2.1 c.c.	16.5 c.c.	14.4 c.c.	
20 mg. leucoprotease + substrate + 2.5 c.c. sp. fl. ....	3.4 c.c.	19.8 c.c.	16.4 c.c.	None

This fluid shows no digestive activity; the increase in digestion in the flask containing spinal fluid and added leucoprotease is difficult to explain. Perhaps the spinal fluid forms, as a result of slightly increased alkalinity, or some other cause, a favorable medium for the action of leucoprotease.

CASE III.—The fluid used in this experiment was obtained after traumatism. It was clear and limpid, of very faint yellow tinge, and showed no sediment. The bacteriological examination was negative. The amount of fluid was 20 c.c.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
0.5 c.c. sp. fl. + substrate.....	1.2 c.c.	1.45 c.c.	0.25 c.c.
1.0 c.c. sp. fl. + substrate.....	1.35 c.c.	1.5 c.c.	0.15 c.c.
2.5 c.c. sp. fl. + substrate.....	1.7 c.c.	1.7 c.c.	None

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ....	1.7 c.c.	14.7 c.c.	13.0 c.c.	
20 mg. leucoprotease + substrate + 0.5 c.c. sp. fl. ....	1.8 c.c.	14.5 c.c.	12.7 c.c.	0.3 c.c.
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.9 c.c.	13.6 c.c.	11.7 c.c.	1.3 c.c.
20 mg. leucoprotease + substrate + 2.5 c.c. sp. fl. ....	2.2 c.c.	16.35 c.c.	14.1 c.c.	None

This fluid apparently exhibits slight power to inhibit the activity of leucoprotease. Absence of red blood cells showed that it con-

tained no blood serum as a result of injury due to tapping. The result indicating inhibition is at variance with the other observations; in the flask containing the largest quantity of spinal fluid, 2.5 c.c., no inhibition is manifest.

The foregoing study of normal spinal fluid shows that this fluid differs markedly in enzymotic and anti-enzymotic content from the blood serum and lymph, for both of these fluids contain an anti-enzyme which is able to check completely the activity of the proteolytic enzyme of the polymorphonuclear leucocyte. The blood serum, moreover, contains an enzyme which is active in acid medium. Tests of the spinal fluid made in Case II above show it to contain no enzyme which is able to cause proteolysis either in acid, neutral or alkaline medium. Furthermore, it does not exhibit the anti-enzymotic activity of the blood and lymph. The absence of both of these qualities is, perhaps, explained, in part, by the low protein content, since it has been demonstrated that the enzymotic activity of the blood is associated with the globulin fraction of the serum, and the anti-enzyme with the albumin. Albumin is entirely wanting in normal spinal fluid. What is known concerning the albumin and globulin of the spinal fluid in pathological conditions suggests the possibility that both enzyme and anti-enzyme may be increased so that in some instances active proteolysis may occur, whereas in others the anti-enzyme may predominate. Doubtless there is some selective power exerted upon the substances which are allowed to pass into the spinal fluid. The almost unique absence of serum albumin in normal fluid suggests this possibility.

Through the kindness of Dr. L. Emmett Holt a specimen of spinal fluid was received from a child suffering with so-called serous meningitis. The etiology of the disease is obscure. Quincke has separated the condition from the ordinary forms of meningitis of known infectious origin. Although in the present instance an autopsy was not obtained the clinical picture was that described as serous meningitis.

CASE IV.—The spinal fluid was clear and limpid and contained no sediment or blood. The volume was 60 c.c. The bacteriological examination was negative.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic . . . . .	1.1 c.c.	1.3 c.c.	0.2 c.c.
1.0 c.c. sp. fl. + substrate (neutral) . . . . .	1.1 c.c.	1.5 c.c.	0.4 c.c.
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb. . . . .	1.1 c.c.	1.2 c.c.	0.1 c.c.

*Anti-enzymotic Activity.*

	Control	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate . . . . .	1.2 c.c.	19.8 c.c.	18.6 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. . . . .	1.2 c.c.	20.3 c.c.	19.1 c.c.	None

In this experiment the fluid examined manifests the absence of enzymotic and anti-enzymotic action previously observed in normal spinal fluid. The lack of anti-enzyme, in this instance, is evidence that the fluid does not contain blood serum. It suggests the absence of a chronic inflammatory condition of the meninges for it will be shown later that, in meningitis running a chronic course, there is a tendency for the anti-enzyme of the spinal fluid to increase.

SUPPURATIVE MENINGITIS.

In the following five cases microorganisms other than *Diplococcus intracellularis meningitidis* of Weichselbaum were isolated from the spinal fluid.

CASE V.—The spinal fluid used in the following test was from a case of suppurative meningitis due to infection with *Streptococcus mucosus*. The course of the disease was acute; the patient, a woman fifty-three years old, dying five days after the onset of symptoms. At lumbar puncture, on the fourth day of the disease, the fluid rose in the manometer tube 205 mm. and 40 c.c. were withdrawn. The fluid was turbid and gave a heavy yellowish sediment on standing. The bacteriological examination showed a pure culture of *Streptococcus mucosus*.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
0.5 c.c. spinal fluid + substrate . . . . .	1.1 c.c.	5.2 c.c.	4.1 c.c.
1.0 c.c. spinal fluid + substrate . . . . .	1.3 c.c.	7.8 c.c.	6.5 c.c.
2.5 c.c. spinal fluid + substrate . . . . .	2.0 c.c.	10.4 c.c.	8.4 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate .....	1.5 c.c.	16.9 c.c.	15.4 c.c.	
20 mg. leucoprotease + substrate + 0.5 c.c. sp. fl. ....	1.7 c.c.	18.3 c.c.	16.6 c.c.	None
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.9 c.c.	18.0 c.c.	16.1 c.c.	None
20 mg. leucoprotease + substrate + 2.5 c.c. sp. fl. ....	2.6 c.c.	17.5 c.c.	14.9 c.c.	0.5 c.c.

There has been active digestion of coagulated protein by this spinal fluid. If anti-body of the blood serum has been poured out into the exudate it is insufficient to check the proteolytic activity of the free enzyme present. There is increased digestion rather than inhibition of leucoprotease with 0.5 and 1.0 c.c. of spinal fluid. Slight inhibition with a greater quantity is difficult to explain for the same amount alone causes active proteolysis.

CASE VI.—The patient from whom the present specimen was obtained died five days after the onset of symptoms of meningitis. The course of the disease was acute. Autopsy showed the dura mater and pia mater to be thickened. There was fairly abundant purulent exudate over the cortex, especially in the sulci and median line. Sections showed that the meninges were covered with a thick layer of fibrin and were pervaded with leucocytes. There was some slight leucocytic infiltration of the cortex in places. At lumbar puncture, performed on the third day of the disease, the fluid flowed out at the top of a manometer tube 550 mm. in length; 100 c.c. were withdrawn. The spinal fluid was pale yellow in color and showed a heavy sediment. *Diplococcus lanceolatus* was isolated in pure culture.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	1.55 c.c.	2.5 c.c.	0.95 c.c.
1.0 c.c. sp. fl. + substrate (neutral) .....	1.55 c.c.	6.1 c.c.	4.55 c.c.
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb...	1.55 c.c.	7.5 c.c.	5.95 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ..	1.65 c.c.	15.75 c.c.	14.1 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	2.15 c.c.	16.7 c.c.	14.55 c.c.	None

Since digestion is much greater in neutral, and particularly in alkaline medium, than in acid, the active enzyme present is the leucoprotease of the polymorphonuclear cells, doubtless set free by their disintegration.

CASE VII.—The patient from whom this specimen was withdrawn was a girl ten years of age. The course of the disease was very acute, the patient dying the day after admission to the hospital. Lumbar puncture on the second day of the disease yielded twenty cubic centimeters of pale yellow, turbid fluid under moderate pressure. On standing a heavy sediment was deposited. The bacteriological examination showed a pure culture of *Diplococcus lanceolatus*.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate.....	1.75 c.c.	7.6 c.c.	5.85 c.c.
2.5 c.c. sp. fl. + substrate.....	2.7 c.c.	11.7 c.c.	9.0 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ...	1.2 c.c.	15.6 c.c.	14.4 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.85 c.c.	17.3 c.c.	15.45 c.c.	None
20 mg. leucoprotease + substrate + 2.5 c.c. sp. fl. ....	2.8 c.c.	18.9 c.c.	16.1 c.c.	None

The spinal fluid digests protein actively. It also increases the activity of leucoprotease. The nitrogen of the incoagulable protein in 5 c.c. of spinal fluid is equivalent to 3.3 cubic centimeters *N/10* sulphuric acid. Its contrast with the normal value of 1.3 c.c. *N/10* sulphuric acid the average from three normal fluids (Cases I, II and III), shows that proteolysis was probably in progress during life in the spinal fluid.

CASE VIII.—In the patient from whom this fluid was obtained, a man thirty-two years old, the involvement of the meninges was secondary to an attack of lobar pneumonia. The duration of the meningitis was two days. The patient died on the seventh day of the disease. On the day of development of meningeal symptoms, 25 c.c. of pale yellow, turbid spinal fluid under slightly increased pressure were obtained. No blood was present in the fluid. The bacteriological examination showed *Diplococcus lanceolatus*.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate (neutral) .....	1.4 c.c.	1.1 c.c.	None
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb....	1.4 c.c.	3.7 c.c.	2.3 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate .....	1.3 c.c.	17.3 c.c.	16.0 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.5 c.c.	17.9 c.c.	16.4 c.c.	None

This fluid shows no digestion in neutral medium, and slight digestion in alkali; there is no inhibition of leucoprotease.

CASE IX.—The patient, a woman twenty-five years old, first showed symptoms of meningitis three weeks before admission to the hospital. These cleared up somewhat, only to become acute again two days before admission. The patient died three days later. Lumbar puncture was done on what was probably the twenty-second day of the disease. Sixty cubic centimeters of cloudy fluid were withdrawn. A moderate yellow sediment was deposited on standing. The fluid showed a blood-stained coagulum and some red blood cells. *Diplococcus lanceolatus* was isolated in pure culture.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic . . . . .	1.3 c.c.	1.7 c.c.	0.4 c.c.
1.0 c.c. sp. fl. + substrate (neutral) . . . . .	1.3 c.c.	1.7 c.c.	0.4 c.c.
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb. . . . .	1.3 c.c.	1.9 c.c.	0.6 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate . . . . .	1.8 c.c.	17.0 c.c.	15.2 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. . . . .	1.9 c.c.	15.9 c.c.	14.0 c.c.	1.2 c.c.

The course of the disease was more extended in this case than in the four preceding cases. There is practically no digestion and only slight inhibition. A few hours before the fluid used was withdrawn an attempt at lumbar puncture had resulted in considerable hemorrhage and it is probable that the spinal fluid obtained later was mixed with blood serum.

The following spinal fluids are from patients suffering with epidemic cerebro-spinal meningitis. All these patients were treated by injection of Flexner's anti-meningitis serum into the subdural space. Specimens of the fluid were obtained both before and after injection through the kindness of Dr. E. Singer of Lebanon Hospital of New York.

CASE X.—The patient from whom spinal fluid was secured was a boy twelve years of age. The course of the disease was mild, the temperature returning to normal on the tenth day. The first lumbar puncture was made on the third day of the disease and the fluid obtained was used in the following experiment. The spinal fluid was pale yellow in color and showed a fairly heavy yellow sediment. No blood was present. *Diplococcus intracellularis meningitidis* was demonstrated in smears and by culture.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	1.3 c.c.	1.8 c.c.	0.5 c.c.
1.0 c.c. sp. fl. + substrate (neutral) .....	1.3 c.c.	1.8 c.c.	0.5 c.c.
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb....	1.3 c.c.	2.1 c.c.	0.8 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ..	1.6 c.c.	15.75 c.c.	14.15 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.85 c.c.	15.3 c.c.	13.45 c.c.	0.7 c.c.

Trivial digestion and inhibition have occurred, and indicate the probable presence of free enzyme and anti-enzyme in very small amounts.

The second lumbar puncture in this case was made on the fourth day of the disease. Thirty cubic centimeters of spinal fluid (*second specimen*) were withdrawn.

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	1.4 c.c.	1.7 c.c.	0.3 c.c.
1.0 c.c. sp. fl. + substrate (neutral) .....	1.4 c.c.	1.4 c.c.	None
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb...	1.4 c.c.	1.45 c.c.	None

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ..	1.6 c.c.	15.75 c.c.	14.15 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.95 c.c.	10.55 c.c.	8.6 c.c.	5.55 c.c.

This fluid causes no digestion, whereas the power of inhibition has become very marked.

Immediately after the last lumbar puncture 30 c.c. of anti-meningitis serum were injected into the subdural space. Thirty cubic centimeters of spinal fluid (*third specimen*) were withdrawn twenty-four hours later on fifth day of disease, preparatory to another injection of serum.

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	1.65 c.c.	1.65 c.c.	None
1.0 c.c. sp. fl. + substrate (neutral) .....	1.65 c.c.	1.9 c.c.	0.25 c.c.
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb..	1.65 c.c.	1.75 c.c.	0.1 c.c.

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ...	1.6 c.c.	15.75 c.c.	14.15 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	2.2 c.c.	12.8 c.c.	10.6 c.c.	3.55 c.c.

Inhibition has diminished somewhat, notwithstanding the injection of 30 c.c. of a serum whose power to inhibit leucoprotease is equal to that of fresh blood serum.<sup>13</sup>

Twenty-four hours before the next specimen of fluid was withdrawn, the patient had received a second injection of 30 c.c. of serum into the subdural space. By lumbar puncture 30 c.c. of cloudy, faintly orange-colored fluid (*fourth specimen*) were secured on the seventh day of the disease.

	Control.	After 5 days at 37°.	Digestion.	
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	1.3 c.c.	1.5 c.c.	0.2 c.c.	
1.0 c.c. sp. fl. + substrate (neutral) .....	1.3 c.c.	1.5 c.c.	0.2 c.c.	
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb....	1.3 c.c.	1.6 c.c.	0.3 c.c.	
	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ...	1.65 c.c.	15.5 c.c.	13.85 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.85 c.c.	16.0 c.c.	14.15 c.c.	None

The anti-enzymotic action of the fluid previously observed has entirely disappeared.

Thirty cubic centimeters of fluid (*fifth specimen*) were withdrawn twenty-four hours after the third injection of 30 c.c. of serum on the eighth day of the disease.

	Control.	After 5 days at 37°.	Digestion.	
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	1.45 c.c.	1.5 c.c.	None	
1.0 c.c. sp. fl. + substrate (neutral) .....	1.45 c.c.	1.4 c.c.	None	
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb....	1.45 c.c.	0.9 c.c.	None	
	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ...	1.9 c.c.	15.75 c.c.	13.85 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	2.0 c.c.	16.0 c.c.	14.0 c.c.	None

During the time represented by the above series of fluids the patient has received into the sub-dural space 90 c.c. of anti-meningitis serum in doses of 30 c.c. at intervals of twenty-four hours. At no time is any digestive action manifest. The inhibitory power of the fluid is greatest on the fourth day of the disease and then rapidly declines to disappear completely on the sixth day of the disease. This loss of inhibition appears in spite of repeated injec-

<sup>13</sup> See page 730.

tions of a serum capable of inhibiting the action of leucoprotease, for previous to the performance of the above series of experiments, tests of the power of the anti-meningitis serum used to inhibit leucoprotease have been made. The following test shows that the serum, after standing on ice for three months, has lost none of its initial power to inhibit leucoprotease.

*Anti-enzymotic Activity of Anti-meningitis Serum Three Months Old.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ...	1.7 c.c.	15.1 c.c.	13.4 c.c.	
20 mg. leucoprotease + substrate + 0.5 c.c. anti-serum .....	1.85 c.c.	2.6 c.c.	0.75 c.c.	12.65 c.c.
20 mg. leucoprotease + substrate + 1.0 c.c. anti-serum .....	2.0 c.c.	2.2 c.c.	0.2 c.c.	13.2 c.c.
20 mg. leucoprotease + substrate + 2.5 c.c. anti-serum .....	2.5 c.c.	2.4 c.c.	None	13.4 c.c.

*Anti-enzymotic Activity of Freshly Drawn Anti-meningitis Serum.*

	Control.	At 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate .....	1.7 c.c.	15.1 c.c.	13.4 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. anti-serum .....	2.0 c.c.	2.3 c.c.	0.3 c.c.	13.1 c.c.

From these results it is seen that anti-meningitis serum retains undiminished, after three months, its power to inhibit leucoprotease.

CASE XI.—The patient from whom the following spinal fluids were withdrawn was a woman twenty-six years old. The course of the disease was severe, the patient dying on the thirteenth day. The first fluid was withdrawn on the tenth day of the disease. It was colorless and contained only a small amount of sediment, and no blood. *Diplococcus intracellularis meningitidis* was isolated from the fluid by culture.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion
1.0 c.c. sp. fl. + substrate.....	1.35 c.c.	1.3 c.c.	None

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ....	1.45 c.c.	10.9 c.c.	9.45 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.6 c.c.	11.1 c.c.	9.5 c.c.	None

No enzymotic or anti-enzymotic action is manifested by this fluid.

The fluid (*second specimen*) obtained the next day resembled that of the first tap. Tests with the quantities previously used gave the following results:

*Enzymotic Activity*.—Digestion: 0.2 c.c.

*Anti-enzymotic Activity*.—Inhibition: 0.7 c.c.

The following specimen of fluid (*third specimen*) was secured on the twelfth day of the disease. During the preceding forty-eight hours the patient had received by injection into the sub-dural space two doses of anti-meningitis serum of 40 c.c. each. The present fluid was orange-yellow in color and contained a heavy sediment. A few red blood cells were present.

*Enzymotic Activity*.—Digestion: none.

*Anti-enzymotic Activity*.—Inhibition: 1.95 c.c.

The fluid shows some inhibition of leucoprotease.

The following test was made with spinal fluid (*fourth specimen*) removed twenty-four hours after the third consecutive injection of 40 c.c. of anti-meningitis serum. The patient died the same day. The fluid was orange-yellow in color. The sediment was less than in the preceding specimen. No blood was present.

*Enzymotic Activity*.—Digestion: 0.1 c.c.

*Anti-enzymotic Activity*.—Inhibition: 1.85 c.c.

The fluids of this series fail to show any digestive power. The inhibitory action upon leucoprotease has been greatest after the second injection of anti-meningitis serum.

CASE XII.—The following series of fluids was obtained from a girl nine years old. The course of the disease was severe and especially resistant to treatment. The patient died on the forty-seventh day of the disease. A record of the amount of fluid withdrawn at each puncture was not kept. The routine procedure in such cases of epidemic meningitis has been to withdraw a volume of spinal fluid equal to the quantity of anti-meningitis serum injected. The first lumbar puncture was made on the first day of the disease. The fluid obtained was turbid and showed a moderate amount of sediment. The centrifugalized specimen was colorless and no red blood cells present. *Diplococcus intracellularis meningitidis* was isolated by culture.

#### *Enzymotic Activity.*

	Control	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	1.1 c.c.	1.5 c.c.	0.4 c.c.
1.0 c.c. sp. fl. + substrate (neutral) .....	1.1 c.c.	1.15 c.c.	None
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb...	1.1 c.c.	1.4 c.c.	0.3 c.c.

#### *Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion	Inhibition.
20 mg. leucoprotease + substrate ...	1.6 c.c.	15.75 c.c.	14.15 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.6 c.c.	10.9 c.c.	9.3 c.c.	4.85 c.c.

The fluid shows practically no digestion and very marked inhibition of leucoprotease.

The *second specimen* of fluid was withdrawn on the second day of the disease. It was slightly turbid, blood-tinged and showed a reddish-yellow sediment.

<i>Enzymotic Activity.</i>				
	Control.	After 5 days at 37°	Digestion.	
1.0 c.c. sp. fl. + substrate .....	1.2 c.c.	1.2 c.c.	None	
<i>Anti-enzymotic Activity.</i>				
	Control.	After 5 days at 37°	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ...	1.65 c.c.	15.5 c.c.	13.85 c.c.	
20 mg. leucoprotease + substrate				
+ 1.0 c.c. sp. fl. ....	1.75 c.c.	14.4 c.c.	12.65 c.c.	1.2 c.c.

The inhibition is less than that determined by the previous test.

The fluid (*third specimen*) used in the following test was obtained on the third day of the disease, and twenty-four hours after the first injection of 30 c.c. of anti-meningitis serum. It was pale orange in color, turbid, and contained a small blood-stained coagulum. More red blood cells were present in this than in the preceding specimens. Quantities used in the foregoing test gave the following results:

*Enzymotic Activity.*—Digestion: none.

*Anti-enzymotic Activity.*—Inhibition: 0.85 c.c.

The fluid (*fourth specimen*) drawn twenty-four hours after the second consecutive injection of 30 c.c. of anti-meningitis serum was pale yellow in color and showed less sediment than the last specimen. No blood was present.

*Enzymotic Activity.*—Digestion: 0.95 c.c.

*Anti-enzymotic Activity.*—Inhibition: 0.35 c.c.

This specimen has caused slight digestion. It might be expected that the inhibitory power of the spinal fluid would increase after the injection of such large quantities of an actively inhibiting serum; instead there is a rapid diminution, terminating on the fourth day of the disease in practically complete loss of the initial anti-enzymotic action.

A *second series of tests* is separated from the preceding series by an interval of six weeks. In the interim the patient had received into the subdural space 345 c.c. of anti-meningitis serum at varying intervals. Seven days before the withdrawal of the following specimen there had been an injection of 30 c.c. of serum. The fluid used for the following test was colorless and only slightly turbid, and contained no blood. It was obtained on the forty-first day of the disease.

*Enzymotic Activity.*—Digestion: none.

*Anti-enzymotic Activity.*—Inhibition: 0.7 c.c.

There is no digestion and only trivial inhibition.

The *second specimen of the series*, removed on the forty-second day of the disease, and twenty-four hours after an injection of 30 c.c. of anti-meningitis serum, was orange-yellow in color and slightly turbid. No blood was present.

*Enzymotic Activity.*—Digestion: none.

*Anti-enzymotic Activity.*—Inhibition: 0.5 c.c.

The fluid withdrawn twenty-four hours later, after the injection of 40 c.c. of anti-meningitis serum, showed no change from the preceding specimen.

*Enzymotic Activity.*—Digestion: none.

*Anti-enzymotic Activity.*—Inhibition: 1.55 c.c.

The following fluid (*third specimen of second series*) was obtained twenty-four hours after the third consecutive injection of anti-meningitis serum (30 c.c.). The fluid was turbid and the orange color due to the pigment of the anti-meningitis serum was fading. No blood was present.

*Enzymotic Activity.*—Digestion: none.

*Anti-enzymotic Activity.*—Inhibition: 0.6 c.c.

The fluid used in the following test (*fourth specimen of this series*) was withdrawn on the forty-fifth day of the disease, and forty-eight hours after the fourth injection of anti-meningitis serum of this series (15 c.c.). It was pale yellow in color and showed more sediment than the preceding specimens. The patient died two days later.

*Enzymotic Activity.*—Digestion: none.

*Anti-enzymotic Activity.*—Inhibition: 0.95 c.c.

At no time represented by these two series of fluids is digestive power manifested. Inhibition of leucoprotease is greatest on the first day of the disease and disappears rather rapidly in the early stages of the disease. The small amount of blood mixed with some of the specimens was too much diluted by the spinal fluid to exhibit any anti-enzymotic activity. This is apparent from the absence of anti-enzyme in the third fluid of the first series of tests in the foregoing experiment. There is slight inhibition on the day after the disease has assumed a somewhat chronic course. The failure of anti-meningitis serum (horse's blood serum) to increase the inhibitory action of the spinal fluid demonstrates that the major portion of this serum is eliminated from the spinal fluid within twenty-four hours after injection.

In view of this disappearance of the anti-enzyme of horse serum from the spinal canal an experiment was performed to determine if the spinal fluid neutralized the anti-enzymotic action of blood

serum. Normal spinal fluid was mixed with varying quantities of fresh beef serum, and the inhibitory action of the mixture tested with leucoprotease. Control digestions were made containing beef serum alone in equal amounts. The presence of spinal fluid did not increase the activity of the anti-enzyme of the serum.

The lack of anti-enzyme in the spinal fluid after the injection of large quantities of horse serum seems then to be due to a rapid elimination of this substance from the spinal fluid. Concerning the rate of absorption of foreign substances from the subdural space, the evidence is somewhat contradictory.

The acute infections of the meninges, which have been studied, represent the common suppurative conditions of the membranes of the brain and spinal cord. The microörganism causing the disease in four instances was *Diplococcus lanceolatus*; in one instance *Streptococcus mucosus*, and in three, *Diplococcus intracellularis meningitis*. Of these eight conditions the spinal fluids in four contained free proteolytic enzyme. In the remaining instances free enzyme, in appreciable quantities, was not demonstrable, and some of the specimens of fluid obtained exhibited anti-enzymotic action. In infections with *Diplococcus lanceolatus*, and with *Streptococcus mucosus*, there has seemed to be a tendency for free enzyme to accumulate in the spinal fluid inasmuch as four of the fluids examined in these conditions have exhibited active proteolysis. This is probably not characteristic of such infections, but is simply an index of the virulence of the infecting microörganism, and the greater severity of the inflammatory reaction. Emigration and disintegration of polymorphonuclear leucocytes proceed so rapidly that the ordinary measure for ridding the spinal fluid of foreign constituents is insufficient. The condition now resembles that in an abscess cavity in which there is abundance of free enzyme.

The results obtained in the series of fluids from the three cases of epidemic meningitis have considerable interest. At no time was it possible to demonstrate any enzymotic activity, though sediment containing polymorphonuclear leucocytes was abundant in many of the specimens of fluid studied. In the fluids withdrawn during the early stages of the disease anti-enzyme was present. This anti-enzymotic action, however, underwent rapid diminution and was

not restored by the injection of large quantities of horse serum, capable of active inhibition of the enzyme employed for the tests. At the same time the amount of cells decreases, so that the loss of anti-enzymotic action can hardly be attributed to the neutralization of anti-enzyme by an increased amount of free enzyme. With the loss of anti-enzyme one would expect the appearance of proteolysis, but at no time did these fluids show digestion. It is probable that both substances are rapidly eliminated from the spinal fluid and are demonstrable only in acute infections when enzyme or anti-enzyme accumulates with great rapidity. The study of inhibition of leucoprotease after injections of anti-meningitis serum (horse's blood serum) showed that the anti-enzyme of the injected serum had disappeared within twenty-four hours from the subdural fluid, even though large amounts of serum had been injected.

#### TUBERCULOUS MENINGITIS.

The spinal fluid in tuberculous meningitis contains few cells, the majority of which are lymphocytes. In most instances serum albumin is present and the total protein is increased in amount. One would expect, therefore, the fluid to manifest no proteolysis and a varying degree of anti-proteolytic action upon the enzyme of polymorphonuclear leucocytes.

CASE XIII.—The spinal fluid used in the following test was from a child two years old. The duration of the disease was not determined. The patient died five days after admission to the hospital. The spinal fluid, obtained by lumbar puncture, was clear and limpid and contained a small coagulum. No blood was present. Bacteriological examination demonstrated the presence of tubercle bacilli in the fluid.

#### *Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
0.5 c.c. sp. fl. + substrate.....	1.0 c.c.	1.0 c.c.	None
1.0 c.c. sp. fl. + substrate.....	1.0 c.c.	1.3 c.c.	0.3 c.c.

#### *Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ...	1.6 c.c.	16.6 c.c.	15.0 c.c.	
20 mg. leucoprotease + substrate				
+ 0.5 c.c. sp. fl. ....	1.6 c.c.	17.3 c.c.	15.7 c.c.	None
20 mg. leucoprotease + substrate				
+ 1.0 c.c. sp. fl. ....	1.6 c.c.	14.7 c.c.	13.1 c.c.	1.9 c.c.
20 mg. leucoprotease + substrate				
+ 2.5 c.c. sp. fl. ....	1.6 c.c.	15.35 c.c.	13.75 c.c.	1.25 c.c.

The fluid shows no digestion and slight inhibition of leucoprotease.

CASE XIV.—The history of the patient in this case could not be secured; the clinical diagnosis was tuberculous meningitis. The spinal fluid appeared clear and colorless and showed a small whitish sediment on centrifugalization. No blood was present.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
0.5 c.c. sp. fl. + substrate.....	1.3 c.c.	1.4 c.c.	0.1 c.c.
1.0 c.c. sp. fl. + substrate.....	1.6 c.c.	1.4 c.c.	None

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate .....	1.6 c.c.	14.9 c.c.	13.3 c.c.	
20 mg. leucoprotease + substrate + 0.5 c.c. sp. fl. ....	1.9 c.c.	13.3 c.c.	11.4 c.c.	1.9 c.c.
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	2.2 c.c.	14.0 c.c.	11.8 c.c.	1.5 c.c.

There is no digestion and slight inhibition of proteolysis.

CASE XV.—Fluid was obtained from a boy five years old. The duration of the disease, as far as could be determined, was about two weeks. There was some evidence of pulmonary tuberculosis. The patient died a few days after admission to the hospital. Although the bacteriological examination was negative, the symptoms, namely, headache, vomiting and convulsions, pointed to tuberculous meningitis. Thirty cubic centimeters of clear spinal fluid were secured by lumbar puncture on the seventh day of the disease. It contained a small coagulum; but no sediment, or blood.

*Enzymotic Activity.*

	Control	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	1.3 c.c.	1.6 c.c.	0.3 c.c.
1.0 c.c. sp. fl. + substrate (neutral) .....	1.3 c.c.	1.2 c.c.	None
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb....	1.3 c.c.	1.3 c.c.	None

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition
20 mg. leucoprotease + substrate ...	1.65 c.c.	15.1 c.c.	13.45 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.85 c.c.	14.2 c.c.	12.35 c.c.	1.1 c.c.

There is no digestion and slight inhibition of leucoprotease.

CASE XVI.—Spinal fluid was obtained from a baby eighteen months old, presenting the ordinary symptoms of tuberculous meningitis. The patient died, as nearly as could be estimated, on the twentieth day of the disease. The spinal

fluid obtained on the tenth day of the disease was pale yellow in color and contained a small coagulum showing many polymorphonuclear and mononuclear cells. No blood was present. The amount withdrawn was thirty cubic centimeters. Tubercle bacilli were demonstrated by bacteriological examination.

*Enzymotic Activity.*

	Control.	After 5 days at 37°	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic . . . .	1.2 c.c.	1.5 c.c.	0.3 c.c.
0.5 c.c. sp. fl. + substrate (neutral) . . . . .	1.15 c.c.	1.2 c.c.	None
1.0 c.c. sp. fl. + substrate (neutral) . . . . .	1.2 c.c.	1.3 c.c.	0.1 c.c.
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb. .	1.2 c.c.	1.4 c.c.	0.2 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate . . .	1.65 c.c.	15.1 c.c.	13.45 c.c.	
20 mg. leucoprotease + substrate + 0.5 c.c. sp. fl. . . . .	1.7 c.c.	13.1 c.c.	11.4 c.c.	2.05 c.c.
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. . . . .	1.75 c.c.	12.1 c.c.	10.35 c.c.	3.1 c.c.
20 mg. leucoprotease + substrate + 2.5 c.c. sp. fl. . . . .	1.9 c.c.	9.6 c.c.	7.7 c.c.	5.75 c.c.

This fluid exhibits practically no digestion, but shows a marked power to inhibit the action of leucoprotease.

CASE XVII.—In the case of the patient, a man thirty years of age, from whom this fluid was withdrawn, a diagnosis of tuberculosis of the lumbar vertebrae, and of the spinal cord had been made. Twenty cubic centimeters of clear fluid were secured by lumbar puncture. A few polymorphonuclear leucocytes and red blood cells were present. The presence of tubercle bacilli was not demonstrated.

*Enzymotic Activity.*

	Control.	After 5 days at 37°	Digestion.
1.0 c.c. sp. fl. + substrate. . . . .	0.95 c.c.	1.0 c.c.	None

*Anti-enzymotic Activity.*

	Control	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate . . .	1.35 c.c.	12.9 c.c.	11.55 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. . . . .	1.5 c.c.	9.3 c.c.	7.8 c.c.	3.75 c.c.

The fluid shows well-marked inhibition of leucoprotease.

CASE XVIII.—Fluid was secured from a boy two years old, exhibiting the ordinary symptoms of tuberculous meningitis. The spinal fluid on about the fourteenth day of the disease was colorless, slightly opalescent, and contained a minute brownish sediment. The presence of tubercle bacilli was demonstrated by bacteriological examination.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	1.2 c.c.	1.4 c.c.	0.2 c.c.
1.0 c.c. sp. fl. + substrate (neutral) .....	1.2 c.c.	1.1 c.c.	None
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb....	1.2 c.c.	1.3 c.c.	0.1 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate .....	1.2 c.c.	19.8 c.c.	18.6 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.3 c.c.	19.8 c.c.	18.5 c.c.	0.1 c.c.

The spinal fluid in this test shows neither digestion nor inhibition.

CASE XIX.—The patient from whom this fluid was obtained was a baby eight months old. The duration of the disease was about one month. The specimen of fluid used in this test was obtained on the twenty-first day of the disease. The spinal fluid, 70 c.c. in amount, was clear and of a pale yellow color. It contained neither blood nor sediment. Bacteriological examination showed the presence of tubercle bacilli.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate.....	1.0 c.c.	1.2 c.c.	0.2 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate .....	0.9 c.c.	19.0 c.c.	18.1 c.c.	
20 mg. leucoprotease + substrate + 0.5 c.c. sp. fl. ....	1.0 c.c.	19.6 c.c.	18.6 c.c.	None
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.1 c.c.	19.5 c.c.	18.4 c.c.	None
20 mg. leucoprotease + substrate + 2.5 c.c. sp. fl. ....	1.4 c.c.	20.5 c.c.	19.1 c.c.	None

The fluid shows no digestion and no inhibition.

In five out of seven specimens of fluids, from cases of tuberculous meningitis, anti-enzyme was present in demonstrable quantities. In no instance was it possible to show the presence of active enzyme. In normal spinal fluid the lack of anti-enzyme is explained, probably by the absence of serum albumin, with which is associated the anti-enzyme of the blood. In certain pathological conditions of the meninges albumin makes its appearance in the spinal fluid; and in cases of tuberculous meningitis the protein content of the fluid may

reach one per cent.<sup>14</sup> In tuberculous meningitis with increased amount of protein, there is a limited source of enzyme because of the low cellular content of the spinal fluid. In view of these facts it is not unexpected that the above series of fluids exhibited no enzymotic activity and a varying degree of anti-enzymotic action.

#### SEROUS EFFUSION WITHOUT INFLAMMATION.

The following three specimens of spinal fluid were obtained from patients exhibiting symptoms suggestive of increased pressure within the subdural space.

CASE XX.—Fluid was obtained from a man suffering with carbon monoxide poisoning. At lumbar puncture the fluid rose in the manometer tube 245 mm. Sixty cubic centimeters of clear, colorless fluid were withdrawn. No blood or sediment was present. The bacteriological examination was negative.

#### *Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
0.5 c.c. sp. fl. + substrate.....	1.95 c.c.	1.2 c.c.	0.25 c.c.
1.0 c.c. sp. fl. + substrate.....	1.0 c.c.	1.45 c.c.	0.45 c.c.
2.5 c.c. sp. fl. + substrate.....	1.2 c.c.	1.75 c.c.	0.55 c.c.

#### *Anti-enzymotic Activity.*

	Control.	After 5 days at 37°	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ..	1.5 c.c.	16.9 c.c.	15.4 c.c.	
20 mg. leucoprotease + substrate + 0.5 c.c. sp. fl. ....	1.55 c.c.	15.8 c.c.	14.25 c.c.	1.15 c.c.
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.6 c.c.	16.1 c.c.	14.5 c.c.	0.9 c.c.
20 mg. leucoprotease + substrate + 2.5 c.c. sp. fl. ....	1.8 c.c.	16.85 c.c.	15.05 c.c.	0.35 c.c.

There is slight inhibition of leucoprotease in the tests with 0.5 and 1.0 c.c. of spinal fluid. A larger quantity has produced no appreciable inhibition.

CASE XXI.—The clinical diagnosis in this case was alcoholic delirium and cerebral edema. Lobar pneumonia subsequently developed and the patient died. No autopsy was obtained. At lumbar puncture 25 c.c. of clear, pale yellow fluid was withdrawn. The intradural pressure apparently was not increased. A minute amount of sediment was apparent after centrifugalization. No blood was present. Bacteriological examination was negative.

<sup>14</sup> Pfaundler, *loc. cit.*

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
0.5 c.c. sp. fl. + substrate.....	0.9 c.c.	1.2 c.c.	0.3 c.c.
1.0 c.c. sp. fl. + substrate.....	0.95 c.c.	1.4 c.c.	0.45 c.c.
2.5 c.c. sp. fl. + substrate.....	1.0 c.c.	1.4 c.c.	0.4 c.c.

*Anti-enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ...	1.5 c.c.	16.9 c.c.	15.4 c.c.	
20 mg. leucoprotease + substrate + 0.5 c.c. sp. fl. ....	1.6 c.c.	15.65 c.c.	14.05 c.c.	1.35 c.c.
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	1.7 c.c.	14.05 c.c.	12.35 c.c.	3.05 c.c.
20 mg. leucoprotease + substrate + 2.5 c.c. sp. fl. ....	2.0 c.c.	11.8 c.c.	9.8 c.c.	5.6 c.c.

The marked inhibition that has been found suggests the presence of a considerable amount of serum albumin, and is evidence that anti-enzyme and perhaps other elements of the blood serum have accumulated in the spinal fluid.

CASE XXII.—Fluid was obtained from a patient with chronic nephritis during an acute uremic attack. Seventy cubic centimeters of spinal fluid were withdrawn and the manometer tube indicated a pressure of 155 mm. The fluid was clear and pale orange in color. No sediment or red blood cells were present. The bacteriological examination was negative.

*Enzymotic Activity.*

	Control.	After 5 days at 37°.	Digestion.
1.0 c.c. sp. fl. + substrate + 0.2 % acetic .....	2.5 c.c.	2.9 c.c.	0.4 c.c.
1.0 c.c. sp. fl. + substrate (neutral) .....	2.5 c.c.	2.4 c.c.	None
1.0 c.c. sp. fl. + substrate + 0.2 % sod. carb....	2.5 c.c.	2.5 c.c.	None

*Anti-enzymotic Activity.*

	Control	After 5 days at 37°.	Digestion.	Inhibition.
20 mg. leucoprotease + substrate ..	1.6 c.c.	15.75 c.c.	14.15 c.c.	
20 mg. leucoprotease + substrate + 1.0 c.c. sp. fl. ....	3.05 c.c.	13.2 c.c.	10.15 c.c.	4.0 c.c.

Inhibition of leucoprotease is well-marked. The high figures for nitrogen in the controls containing spinal fluid, is perhaps explained by an increase of urea in this fluid.

Tests of the three fluids here presented represent conditions associated with symptoms of increased pressure within the subdural

space, presumably due to transudation from the blood vessels. In two instances large amounts of fluid were secured at lumbar puncture and the pressure recorded by the manometer tube was high. That elements of the blood serum under such conditions pass into the spinal fluid, and are not efficiently eliminated, is manifest from the power to inhibit leucoprotease exhibited by these fluids.

#### CONCLUSIONS.

The experiments which have been presented show that the spinal fluid occupies a unique position among the fluids which accumulate in serous cavities of the body. It contains normally neither proteolytic enzyme nor anti-enzyme, whereas blood serum, from which it is derived, exhibits both enzymotic and anti-enzymotic activity.

In the blood anti-enzyme greatly predominates over enzyme, so that proteolysis does not occur, unless the anti-enzymotic power of the serum has been destroyed by the addition of acid. In pathological conditions both enzyme and anti-enzyme may make their appearance in the spinal fluid. With inflammations of other serous cavities of the body the anti-enzyme of the exuded serum as a rule preponderates over and restrains the activity of the proteolytic enzyme freed from leucocytes. On the other hand, in infection of the meninges with *Diplococcus lanceolatus* and with *Streptococcus mucosus* free proteolytic enzyme has been present in considerable amount in four of five fluids which have been tested. Free proteolytic enzyme has not been observed in the spinal fluid in cases of epidemic meningitis.

The cases which have been studied demonstrate that in epidemic meningitis some anti-enzymotic action may be present in the early stages of the disease; but it tends to disappear rapidly so that anti-enzyme seems to be constantly at a low ebb. It is possible that the absence of anti-enzyme in normal spinal fluid, and the tendency for it to disappear so much more rapidly than in other inflammatory exudates, may explain in part the severity of acute meningeal infections.

Non-inflammatory transudates into the subdural spaces differ from inflammatory exudates in that the inhibitory element of the blood serum accumulates, and this accumulation suggests an inter-

ference with the elimination of the antibody from the spinal fluid. Such interference is not evident in so-called serous meningitis.

In content of anti-enzyme the spinal fluid of chronic conditions, such as tuberculous meningitis, apparently occupies an intermediate position between acute inflammation and serous effusion, and five of seven tuberculous fluids which were tested exhibited various degrees of anti-enzymotic action.

Variations in content of enzyme and anti-enzyme, noted above, may depend upon the rapidity with which the fluid, carrying the elements mentioned, enters the spinal cavity, as well as upon the rate of their elimination from the spinal fluid. Subdural injection of large quantities of anti-meningitis serum (horse's blood serum) does not increase the anti-enzymotic activity of fluids withdrawn twenty-four hours after its injection; disappearance of anti-enzyme being caused by rapid elimination of serum from the spinal fluid.

I wish to express my indebtedness to Dr. Opie for the suggestion and supervision of this work. Specimens of spinal fluid were obtained from the Presbyterian Hospital of New York, through the kindness of Dr. J. C. Meakins, and from the Babies Hospital, through the kindness of Dr. Martha Wollstein.





## THE LEUCIN FRACTION IN CASEIN AND EDESTIN.

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An accurate method having been developed for determination of the valin, leucin and isoleucin which compose the "leucin fraction" of the complex proteins, it seemed desirable for comparison to repeat the analyses of this fraction as obtained from one or more proteins which have already been subjected to hydrolysis under what might be regarded as standard conditions according to previous methods. Casein and edestin from flax seed were chosen, because they are, if not definite chemical compounds, at least obtainable by well-fixed methods which should insure products of definite character and composition; and their hydrolyses have been performed by Abderhalden, who has probably had more experience than any other worker in the field of protein hydrolyses.

### *Casein.*

Four hundred and sixty-four grams, calculated ash and moisture free, of Kahlbaum's casein prepared according to Hammarsten, were hydrolyzed by 12 hours' boiling with 25 per cent hydrochloric acid and esterified. The esters were divided into two portions for convenience, freed with barium hydrate, and extracted. The residue was esterified a second time, and another repetition yielded a small third crop of esters.

Because of the extreme convenience of the barium method for freeing the esters,<sup>1</sup> we repeat in some detail the manner in which it is now used in this laboratory. The alcoholic solution of ester hydrochlorides is concentrated *in vacuo* as usual. The great part of the alcohol is driven off, without carrying the concentration so far that the solution is too thick to remove easily from the flask. Keeping the temperature of the bath at 45° to 50° towards the close of the concentration assures this. The concentrated esters are poured into an enamelled jar, of about 2.5 liters

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<sup>1</sup> First used by Levene, this *Journal*, i, p. 4, 1905; Levene and Alsberg: *Ibid.*, ii, p. 128, and further developed by Levene and Van Slyke: *Biochem. Zeitschr.*, xiii, p. 442, 1908.

capacity for the products of 250 grams of protein, and the flask rinsed with a small volume of ice-cold baryta water to which is added powdered barium hydrate. The jar with esters and rinsings is placed in a freezing mixture and stirred with a wooden spatula while pulverized barium hydrate is added rapidly. The mass, at first usually thick, becomes in a few minutes semi-liquid and alkaline. As soon as the alkaline reaction appears the mixture is covered with ice-cold ether, and the stirring continued, anhydrous barium hydrate in portions of 10 to 20 grams being now added at intervals of several minutes. As the fluid water is taken up by the anhydrous baryta the esters are removed by the ether. The residual mass finally becomes sufficiently dry to break into small, easily stirred granules. They still contain enough moisture to hold them together and prevent formation of a suspension of dry powder in the ether. When the granular condition is reached the addition of barium hydrate is discontinued. If this point is passed and a suspension forms, the ether must be filtered on a large Buchner. When the anhydrous baryta is added with moderate care, however, no trouble is experienced in reaching the proper end point. The ice-cold ether is renewed several times during the operation, and the extraction continued until the extracts are colorless. The latter are shaken with potash and dried over sodium sulphate as usual.

In order to make the yield of esters as nearly quantitative as possible, the barium residue is thoroughly trituated several times with water, and finally washed on a Buchner funnel. The washings, which contain the amino-acids not removed by the first esterification and extraction, are quantitatively freed from barium by sulphuric acid, concentrated *in vacuo*, and re-esterified. Extraction as described above yields a second smaller crop of esters, and by repeating the process, a third and even a fourth crop in decreasing yields may be obtained.

The freeing of the esters is really the critical point in the ester method, and the above technique has several advantages over the usual sodium hydrate and potash method. A large excess of free alkali in solution is impossible when barium is used, because the low solubility of barium hydrate in the cold makes its concentration a self-regulating function. For the same reason, the heat of neutralization is generated no faster than it can be removed by the freezing mixture, so that over-heating is avoided and saponification of esters undoubtedly reduced. It is not difficult to keep the temperature of the mixture below 5° while the esters are being freed and extracted, and still finish the process within three quarters of an hour. Another advantage is the ease with which the barium is removed in order to prepare for esterification of the residues. Within a few hours the solution of unextracted amino-acids may be freed from mineral matter, and started concentrating, for a second esterification; while the process of changing masses of sodium hydroxide and potassium carbonate to chlorides and removing them by repeated concentration requires much greater expenditure of time and effort, with increased opportunities for loss of material.

The barium method has also been used with success for small amounts

of esters. In one case 92 per cent of the theoretical yield of constant-boiling valin ester was obtained from 5 grams of valin.

The casein esters were distilled, using sulphuric acid in glass-wool to absorb the gases evolved<sup>1</sup> instead of condensing them with liquid air. The following fractions were obtained:

	Pressure. mm.	Temp. Vapors. degrees.	Wt. of esters grams.
I .....	20	to 65	61.5
II .....	0.3	" 87	165.7
III .....	0.8	" 140	119.7
Total .....			346.9
Undistilled residue .....			63.4

Only fractions I and II were worked up for the leucin fraction

*Fraction I.* The esters yielded 8.28 grams of amino-acids, which were reduced to 6.20 grams by extraction with alcohol to remove protein. Fractional crystallization yielded 2.65 grams of leucin-valin mixture. From this, 1.70 grams of lead-leucin, equivalent to 0.95 gram of leucin, was obtained.

Analysis: 0.3023 gm. substance; 0.1954 gm.  $\text{PbSO}_4$ ; 44.14 per cent Pb.  
Calculated for  $\text{Pb}(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2$ : 44.29 per cent Pb.

The leucin was regenerated, and gave the following rotation in 20 per cent hydrochloric acid:

0.4851 gram of substance; 11.31 grams of total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 0.83°.

$$[\alpha]_D^{20} = +20.3^\circ$$

Calculated from the rotation (cf. p. 400, preceding paper), the substance consisted of 21.6 per cent or 0.20 gram d-isoleucin and 0.75 gram l-leucin.

The filtrate from the lead-leucin yielded 1.63 grams of valin.

Analysis: 0.1221 gram substance; 0.2288 gram  $\text{CO}_2$ ; 0.1045 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C.....	51.24 per cent	51.10 per cent.
H.....	9.47 "	9.75 "

<sup>1</sup> Levene and Van Slyke: *Biochem. Zeitschr.*, x, p. 214, 1908.

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The mother liquors from the above leucin-valin mixture contained mostly alanin, but yielded by crystallization from dilute alcohol 0.50 gram of valin.

Analysis: 0.0975 gram substance; 0.1829 gram  $\text{CO}_2$ ; 0.0857 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C . . . . .	51.24 per cent	51.17 per cent.
H . . . . .	9.47 "	9.82 "

*Fraction II.* The esters were saponified with about 800 cc. of water, and the solution left over night to complete crystallization. 38.11 grams of leucin (crop *a*) accompanied by a small amount of valin were obtained. The substance showed, on analysis, 54.46 per cent C, 9.80 per cent H, instead of 54.92 per cent C and 9.99 per cent H. Estimated from the analysis, the fraction should contain 33.4 grams of leucin isomers, 4.7 grams of valin. Twenty grams were submitted to the lead separation, the leucin being removed by two precipitations (cf. p. 396, preceding paper), yielding respectively 27.20 and 4.51 grams of lead-leucin, equivalent together to 17.80 grams of leucin.

Analyses: (1st ppt.) 0.3056 gram substance; 0.1973 gram  $\text{PbSO}_4$ ; 44.10 per cent Pb.

(2d ppt.) 0.3276 gram substance; 0.2120 gram  $\text{PbSO}_4$ ; 44.19 per cent Pb.

Calculated for  $\text{Pb}(\text{C}_6\text{H}_{11}\text{O}_2\text{N})_2$ : 44.29 per cent Pb.

The leucin was regenerated from the lead salt.

Analysis: 0.1134 gram substance; 0.2279 gram  $\text{CO}_2$ ; 0.1021 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$	Found:
C . . . . .	54.92 per cent	54.80 per cent.
H . . . . .	9.99 "	10.07 "

Rotation in 20 per cent hydrochloric acid: 0.6984 gram substance; 18.994 grams total solution; observed rotation + 1.32°.

$$[\alpha]_D^{20} = + 17.23^\circ$$

From the rotation:

$$\text{Isoleucin} = 100 \times \frac{17.23 - 15.6}{21.8} = 7.5 \text{ per cent; leucin} = 92.5 \text{ per cent.}$$

The filtrate from the second precipitate of lead-leucin yielded 1.77 grams of valin.

Analysis: 0.1286 gram substance; 0.2403 gram  $\text{CO}_2$ ; 0.1086 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_9\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C.....	51.24 per cent	50.94 per cent.
H.....	9.47 "	9.44 "

Rotation in 20 per cent hydrochloric acid: 0.4800 gram substance; 22.360 grams total solution; + 1.08° rotation in 1.894 dm. tube; sp. gr., 1.10.

$$[\alpha]_D^{20} = + 24.2^\circ$$

The alcoholic filtrate from the valin yielded a slight amount of brown syrup, similar in appearance to crude prolin.

The above yields indicate for the total 38.11 grams, constituting the first crop of crystals, 31.37 grams of leucin, 2.54 grams of isoleucin, 3.37 grams of valin, 0.83 gram not recovered, probably largely prolin.

A second crop (b) of amino-acids, weighing 40.63 grams, was obtained by concentrating the filtrate from the first *in vacuo* to a small volume, and washing the product with 60 per cent alcohol. Analysis showed 52.08 per cent C, 9.60 per cent H, indicating 9 grams of leucin in the substance (8.7 grams were found). The acids did not have the appearance of the typical leucin-valin mixture, probably because some prolin was present. Allowance was erroneously made for the possible effect of alanin on the analysis, and enough lead to precipitate 17 grams of leucin (65 cc.) used in the separation. As a result the lead precipitate contained some valin. The lead content was high and the regenerated acids, 11.45 grams, gave 54.03 to 54.01 per cent C, and 9.85 to 9.91 per cent H, indicating that they were one-fourth valin. 8.96 grams were dissolved in 60 cc. water plus 10 cc. ammonia, and precipitated with 28 cc.  $\frac{M}{2}$  lead acetate, equivalent to 7.34 grams of leucin. 11.20 grams of lead leucin were obtained. The filtrate was concentrated *in vacuo* to about 30 cc. and 3 cc. concentrated ammonia added, precipitating 0.91 gram more of lead salt, making a total of 12.11 grams, equivalent to 6.80 grams of leucin.

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Analyses: (1st ppt.): 0.3242 gram substance; 0.2111 gram  $\text{PbSO}_4$ ; 44.47 per cent Pb.

(2d ppt.): 0.1966 gram substance; 0.1270 gram  $\text{PbSO}_4$ ; 44.12 per cent Pb.

Calculated for  $\text{Pb}(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2$ , 44.29 per cent Pb.

Analysis of regenerated leucin: 0.1156 gram substance; 0.2321 gram  $\text{CO}_2$ ; 0.1070 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{12}\text{O}_2\text{N}$ :	Found:
C.....	54.92 per cent	54.76 per cent.
H.....	9.99 "	10.36 "

Rotation in 20 per cent hydrochloric acid: 0.7322 gram substance; 19.054 grams total solution; sp. gr., 1.10; rotation in 1.894 dm. tube, + 2.06°.

$$[\alpha]_D^{20} = + 25.85^\circ$$

Calculated from the rotation, the substance is 47.0 per cent isoleucin, 53.0 per cent leucin. The filtrate from the lead-leucin gave 2.00 grams of valin.

Analysis: 0.1453 gram substance; 0.2751 gram  $\text{CO}_2$ ; 0.1247 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C.....	51.24 per cent	51.63 per cent.
H.....	9.47 "	9.60 "

From the above figures, the original 11.45 grams of acids in the first lead precipitate consisted of 4.60 grams of leucin, 4.09 grams of isoleucin, 2.56 grams of valin, 0.20 gram unaccounted for.

Because of the original appearance of crop *b* in this ester fraction, the filtrate from the first lead precipitate was not concentrated to dryness, but reduced *in vacuo* only until crystallization of valin began. About an equal volume of alcohol was added, and the solution left over night in the refrigerator. The crystals were washed with 60 per cent alcohol, and 15.23 grams of valin obtained.

Analysis: (1) 0.1033 gram substance; 0.1944 gram  $\text{CO}_2$ ; 0.0895 gram  $\text{H}_2\text{O}$ .

(2) 0.1523 gram substance; 0.2874 gram  $\text{CO}_2$ ; 0.1316 gram  $\text{H}_2\text{O}$ .

	Calculated for $C_6H_{11}O_2N$ :	Found:
C.....	51.24 per cent	(1) 51.34 per cent. (2) 51.47 "
H.....	9.47 "	(1) 9.68 " (2) 9.67 "

Rotation in 20 per cent hydrochloric acid: 0.4720 gram substance; 11.188 grams total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.10°.

$$[\alpha]_D^{20} = + 27.4^{\circ}$$

To the mother liquors a small amount of alcohol-insoluble amino-acid regained from the mother liquors of main crop *b* was added, and the solution concentrated until crystallization was again well under way, then alcohol added as before. 7.78 grams more of valin was obtained.

Analysis: 0.1324 gram substance; 0.2465 gram  $CO_2$ ; 0.1140 gram  $H_2O$ :

	Calculated for $C_6H_{11}O_2N$ :	Found:
C.....	51.24 per cent	50.78 per cent.
H.....	9.47 "	9.63 "

The valin was once recrystallized by dissolving in hot water and adding alcohol.

Analysis: 0.1306 gram substance; 0.2449 gram  $CO_2$ ; 0.1125 gram  $H_2O$ .

	Calculated for $C_6H_{11}O_2N$ :	Found:
C.....	51.24 per cent	51.16 per cent.
H.....	9.47 "	9.64 "

Rotation in 20 per cent hydrochloric acid: 0.4930 gram substance; 11.403 grams total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.04°.

$$[\alpha]_D^{20} = + 25.3^{\circ}$$

The amino-acids from the mother liquors were freed from prolin with alcohol, and changed to copper salts, of which 4.81 grams were obtained. Analysis indicated alanin-copper, with a small admixture of valin, but the amount of the latter was not sufficient to isolate.

The above analysis indicates, for the 40.63 grams of the second

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crop (b) of crystals, 4.60 grams of leucin, 4.09 grams of isoleucin, 25.57 grams of valin, 6.37 grams loss and other amino-acids.

This makes the total yield of the leucin fraction:

Fraction.	Leucin grams.	Isoleucin. grams.	Valin. grams.
Ia.....	0.75	0.20	1.63
Ib.....	.....	....	0.50
IIa.....	31.37	2.54	3.37
IIb.....	4.60	4.09	25.57
Total.....	36.72	6.63	31.07
Per 100 grams of casein.....	7.92	1.43	6.69

### EDESTIN.

As a combination of direct crystallization and esterification had previously been used with success in obtaining the leucin fraction,<sup>1</sup> particularly from relatively small amounts of protein, this method was employed for edestin. The process is more complicated, but theoretically should yield more nearly the total amount of the leucin fraction than simple esterification, because the amino-acids gained by direct crystallization escape the losses from anhydride formation, incomplete extraction, etc., that may accompany esterification. Its actual applicability, however, appears to vary with the nature of the protein, for the crystallized leucin may carry with it impurities, the removal of which results in more loss than would occur by esterification. This was our experience with casein. As a general method direct esterification, as applied in the preceding casein hydrolysis, seems preferable. In the case of edestin, the leucin and valin from the crystallized acids were not so pure as those obtained from the esters, but they appeared sufficiently so to justify an approximate estimation of their relative amounts in the protein.

Two hundred grams of edestin, calculated ash and moisture free, were hydrolyzed by 12 hours boiling with 25 per cent hydrochloric acid. The hydrochloric acid was partially removed by concentration *in vacuo*, the solution then diluted to 7 liters, and the remainder of the chlorine removed by means of precipitated

<sup>1</sup> Levene and Mandel: *Biochem. Zeitschr.*, v, p. 36, 1907. Levene and Van Slyke: *Ibid.*, xiii, p. 450, 1908.

lead oxide and silver sulphate.<sup>1</sup> The slightly yellow solution obtained was concentrated *in vacuo* until the tyrosin crystallized. 5.34 grams of pure tyrosin needles were obtained. This exceeds somewhat the amount (2.1 grams per 100 grams edestin) found by Abderhalden, and apparently constituted practically all of the tyrosin present. The filtrate gave only a slight Millon reaction, and no tyrosin needles were observed in succeeding crops of crystals.

The filtrate was concentrated *in vacuo*, and yielded three crops of crystals, weighing 16.20, 7.17, and 3.10 grams respectively. The crystals, washed thoroughly with 70 per cent alcohol, were nearly white and of fairly pure appearance, both microscopically and in mass. The first two crops were dissolved together and, as their composition was uncertain, enough lead added to precipitate 17 grams of leucin. 21.88 grams of lead-leucin, equivalent to 12.28 grams of leucin, were obtained.

Analysis: (1) 0.3169 gram substance; 0.2026 gram  $\text{PbSO}_4$ ; 43.66 per cent Pb.

(2) 0.4689 gram substance; 0.3004 gram  $\text{PbSO}_4$ ; 43.75 per cent Pb.

Calculated for  $\text{Pb}(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2$ . 44.29 per cent Pb.

The crude valin from the filtrate was recrystallized, but could not be obtained perfectly pure. 7.05 grams of the following composition were obtained, the mother liquor being added to the main solution for esterification.

Analysis: 0.1603 gram substance; 0.3045 gram  $\text{CO}_2$ ; 0.1275 gram  $\text{H}_2\text{O}$

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C.....	51.24 per cent	51.81 per cent.
H.....	9.47 "	8.90 "

Rotation in 20 per cent hydrochloric acid: 0.5225 gram substance; 11.124 grams total solution; sp. gr, 1.10; rotation in 0.865 dm. tube, + 1.16°.

$$[\alpha]_D^{20} = + 25.9^\circ$$

The rotation agrees with those found by other authors for natural valin (cf. footnote p. 415, preceding article). The valin was transformed

<sup>1</sup>Levene and Van Slyke: *Biochem. Zeitschr.*, xiii, p. 448, 1908.

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into copper salt, which gave the following figures on combustion: 0.1865 gram substance; 0.2825 gram  $\text{CO}_2$ ; 0.1107 gram  $\text{H}_2\text{O}$ ; 0.0521 gram  $\text{CuO}$ .

	Calculated for $\text{Cu}(\text{C}_6\text{H}_{10}\text{O}_2\text{N})_2$	Found:
C.....	40.57 per cent	41.30 per cent.
H.....	6.64 "	6.83 "
Cu.....	21.50 "	22.30 "

The third crop of crystals gave 2.08 grams of lead salt, equivalent to 1.17 grams of leucin.

Analysis: 0.3161 gram substance; 0.2052 gram  $\text{PbSO}_4$ ; 44.34 per cent Pb.  
Calculated for  $\text{Pb}(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2$ : 44.29 per cent Pb.

From the filtrate by recrystallization 1.02 grams of valin were obtained, of the following composition:

0.1603 gram substance; 0.2967 gram  $\text{CO}_2$ ; 0.1336 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C. . . . .	51.24 per cent	50.47 per cent.
H.....	9.47 "	9.32 "

The mother liquors from the crystals were concentrated and submitted to two esterifications. The esters boiling below  $90^\circ$  at 0.5 mm. were worked up for leucin and valin. The less soluble crystal fractions, containing the leucin fraction, were combined and the leucin precipitated as usual. 4.94 grams of lead-leucin, equivalent to 2.77 grams of leucin, were obtained.

Analysis: 0.3061 gram substance; 0.1991 gram  $\text{PbSO}_4$ ; 44.44 per cent Pb.  
Calculated for  $\text{Pb}(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2$ : 44.29 per cent Pb.

The filtrate gave 3.08 grams of valin.

Analysis: 0.1350 gram substance; 0.2531 gram  $\text{CO}_2$ ; 0.1131 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C.....	51.24 per cent	51.14 per cent.
H.....	9.47 "	9.34 "

Rotation in 20 per cent hydrochloric acid: 0.5102 gram substance; 11.275 grams total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, +  $1.10^\circ$ .

$$[\alpha]_D^{20} = + 25.6^\circ$$

Because the leucin from direct crystallization, obviously from the low lead content (43.7 instead of 44.3 per cent), contained slight amounts of impurities, which might affect the rotation somewhat, the polarimetric determination of the proportion of isoleucin was not applied. The free leucin from all the lead precipitates gave on analysis 54.98 per cent C, 9.30 per cent H, and showed a specific rotation of  $+19.7^\circ$  in 20 per cent hydrochloric acid.

The results are summarized as follows:

	Leucin. grams.	Valin. grams.
Direct crystallization.....	13.45	8.07
Esters.....	2.77	3.08
Total.....	16.22	11.15
Per 100 grams of edestin.....	8.1	5.6

Although the figures are minimal, and doubtless considerably short of the true values, the relative proportions of leucin and valin are, we believe, approximately correct.

A comparison of our figures for the leucin fractions of casein and edestin with the figures of Abderhalden is given below. They represent grams of amino-acid per 100 grams of protein.

	CASEIN.		EDESTIN.	
	Levene and Van Slyke	Abder- halden. <sup>1</sup>	Levene and Van Slyke	Abder- halden. <sup>2</sup>
Leucin.....	7.92	10.5	8.1	20.9
Isoleucin.....	1.43			
Valin.....	6.69	1.0	5.6	0.45
Total leucin fraction.....	16.04	11.5	13.7	20.9

The most striking differences between our results and Abderhalden's are in the valin, which apparently was nearly all calculated in with the leucin by Abderhalden (cf. footnote, p. 393, preceding article). The probability that much more valin was present

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xlv, p. 23.

<sup>2</sup> *Ibid.*, xxxvii, p. 499 (no valin reported); xl, p. 249 (slight amount of valin reported from leucin fraction of previous hydrolysis).

than could be isolated was recognized by Abderhalden.<sup>1</sup> That our total figures for leucin plus valin in edestin are much lower, is probably partly due to the fact that Abderhalden's figures must have been based on crude products, as shown by the fact that but a small fraction of the valin present was separated from the leucin. Our figures, although we do not believe they are as high as might be obtained, are based upon approximately pure substances. Abderhalden's casein figures were doubtless also based on incompletely purified products, but in this case our total yield is 40 per cent larger, although based entirely on analytically pure substances. The difference is probably due to a more complete extraction of the esters freed by the barium method, as our yield was exceptionally high, 347 grams of esters from 464 grams of casein.<sup>2</sup>

Considering the lack of even approximate accuracy in Abderhalden's figures for the leucin fraction, which are taken from what may be considered standard hydrolyses by the methods previously available, it appears probable that the figures for the leucin fraction in most of the protein hydrolyses hitherto published are in need of revision.

<sup>1</sup> "Bis jetzt ist unter den Spaltungsprodukten der Proteine nur d-Valin aufgefunden worden, und zwar meist nur in geringer Ausbeute. Es unterliegt jedoch keinem Zweifel, dass das Valin in Wirklichkeit in grösseren Mengen vorhanden ist. Es zeigt die Neigung, mit -l-Leucin und d-Isoleucin Mischcrystalle zu bilden und ist von diesen nur unter grossen Verlust in völlig reinem Zustande zu trennen." E. Abderhalden, *Neuere Ergebnisse der Eiweisschemie*, p. 38, 1909.

<sup>2</sup> The Dennstedt method was used for the numerous combustions connected with the foregoing work, and was found exceedingly satisfactory.

## THE SERODIAGNOSIS OF SYPHILIS \*

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It is my essential purpose to present the results of the analysis of 2,406 blood serums by the complement fixation test, using either Wassermann's or my own method of doing the test. Herewith are given six tables, giving in detail the results of the analysis and of a comparative study of 244 cases of known syphilitic or parasymphilitic conditions in which the Wassermann method and mine are contrasted. I shall also point out briefly the main differences between the Wassermann method and my own, referring to the various other modifications in passing. For further details consult my article read before the Section on Pathology and Physiology of this Association.

It is important to bear in mind that the main difference between my system and the Wassermann system lies in my use of a known quantity of amboceptor, while in the Wassermann system we meet a difficulty in the fact that human serum may contain anywhere from 0 to 20 units of natural hemolysin (amboceptor) capable of inducing hemolysin of sheep's corpuscles. This introduces an uncertain factor into the interpretation of the results, inasmuch as an excess of anti-sheep amboceptor leads to hemolysis even in the presence of syphilitic antibody. Any system using foreign corpuscles, such as that of Bauer, Hecht, Stern, and Detre, is equally apt to give fallacious results.

Minor differences often of great importance to the individual doing the test are: (1) in my system it is possible to preserve the various reagents, such as antigen, amboceptor, and, if necessary complement, in stable form, dried on filter-paper and then titrated, ready for use; (2) in my system the quantity of blood necessary for the test is very small, only a few drops; inactivation

\* Read in the Section on Nervous and Mental Diseases of the American Medical Association, at the Sixtieth Annual Session, held at Atlantic City, June, 1909.

is not necessary and the blood need not be fresh; (3) the ease with which certain of the reagents can be procured is a factor; in my system human corpuscles are used, and the patient's own cells can be utilized. In all the other methods it is necessary to have a fresh supply of corpuscles, sheep's, horse's, etc., always on hand; (4) all the other methods require a complete laboratory, while any laboratory worker can do my test with a very small equipment.

Table 1 contains a summary of the results obtained by my method. The percentage of positive reactions in these cases of syphilis, manifest or latent, compares favorably with the results obtained by different investigators using the Wassermann system.

TABLE 1.—THE RESULTS OBTAINED BY NOGUCHI'S METHOD IN, SYPHILIS, PARASYPHILIS, HEREDITARY SYPHILIS AND SUSPECTS

	Number of Cases.	+	%	—	+
Primary Syphilis .....	70	65=	92.8	4	1
Secondary Syphilis .....	197	190=	96	5	2
Tertiary Syphilis .....	177	159=	89.9	16	2
Early Latent Syphilis .....	115	87=	75.6	24	4
Late Latent Syphilis .....	150	119=	79.3	27	4
Under Prolonged Treatment .....	39	4=	10.2	32	3
Cerebral Syphilis .....	5	3=	60	1	1
Tabs .....	125	85=	68	27	13
General Paralysis .....	15	13=	86.6	2	0
Hereditary Syphilis .....	17	17=	100	0	0
Suspected Syphilis .....	172	60=	34.8	96	16
	1082	802		234	46

TABLE 2.—RESULTS OBTAINED BY NOGUCHI'S METHOD IN CASES IN WHICH SYPHILIS IS AN ETIOLOGIC FACTOR OR, CAN NOT BE EXCLUDED AS A POSSIBLE CAUSE OF THE CONDITION

	Number of Cases	+	—	+
Cirrhosis of Liver .....	7	5	1	1
Ascitic Fluids .....	21	11	9	0
Aortic Insufficiency .....	1	1	0	0
Chronic Arthritis .....	10	2	6	2
Eye Cases .....	29	14	15	0
Diabetes .....	5	1	4	0
Eczema .....	32	1†	31	0
Scleroderma .....	4	1†	3	0
Brain Tumor? .....	8	4	4	0
Central Gliosis? ..	2	1	1	0
Hemiplegia .....	8	3	5	0
Spastic Paraplegia .....	3	2	0	1
	130	46	81	3

† This serum was sent to me by Dr. Howard Fox, who failed to obtain another specimen from the same case.

† This serum was sent to me by Dr. Schuyler Clark, who made a report on the same case elsewhere.

Table 2 is made up of cases in which syphilis could not be excluded as an etiologic factor. In many cases in which the diagnosis clinically was syphilis, a positive reaction was obtained, and *vice versa*. In the one case of eczema a second specimen was not obtainable; and in

this case the patient could not be further interrogated to obtain, if possible, a history of lues. The possibility of hereditary syphilis in the one case of scleroderma could not be excluded; the mother gave a weak reaction to the test.

The material for Table 3 was supplied by Dr. Martin Cohen. In every case in which a positive reaction was obtained, with exception of the one case of acromegaly, the clinical diagnosis made by Dr. Cohen was syphilis.

TABLE 3.—RESULTS OBTAINED BY NOGUCHI'S METHOD IN EYE CASES

	Number of Cases.	+	—	+
Keratitis Interstitialis .....	12	8	4	0
Iritis (acute) .....	6	4	2	0
Scleritis .....	1	0	1	0
Paralysis Exter. Rectus .....	1	0	1	0
Neuritis Optic .....	1	1	0	0
Chorioiditis Exudative .....	1	0	1	0
Atrophy Optic .....	5	0	5	0
Acromegaly with Ocular Symptoms .....	2	1	1	0
	29	14	15	0

In Table 4 I have reported a number of miscellaneous diseases in which syphilis was probably not a factor. Among these were certain cases of leprosy, malignant neoplasms, tuberculosis, scarlet fever and other exanthemata, in all which diseases positive reactions have been occasionally reported by those using the Wassermann method. It can not be denied that complement is fixed in a large percentage of cases of leprosy; the reasons for this result are as yet unknown. With carcinoma and scarlet fever a positive reaction is rarely obtained. The results here agree closely with those obtained by the Wassermann method.

TABLE 4.—RESULTS OBTAINED BY NOGUCHI'S METHOD IN CASES IN WHICH SYPHILIS CAN BE EXCLUDED WITH A FAIR DEGREE OF CERTAINTY

	Number of Cases	+	—	+
Leprosy .....	10	7 (!)	2	1
Carcinoma .....	51	1	48	2
Sarcoma .....	3	0	3	0
Adenosarcoma .....	1	0	1	0
Endothelioma .....	1	1	0	0
Scarlatina .....	63	1	60	2
Varicella .....	1	0	1	0
Measles .....	2	0	2	0
Tuberculosis .....	52	0	52	0
Lupus .....	2	0	2	0
Raynaud's Disease .....	2	0	2	0
Banti's Disease .....	1	1	0	0
Hodgkin's Disease .....	2	0	2	0
Muscular Dystrophy .....	5	1	3	1
Neurasthenia .....	2	0	1	1
Dementia Præcox .....	5	0	5	0
Various Skin Diseases .....	58	0	57	1
Miscellaneous .....	74	0	74	0
	335	12	315	8

Table 5 presents a study of 615 cases by the regular Wassermann method; and the results obtained agree closely with those of other investigators.

TABLE 5.—RESULTS OBTAINED BY THE WASSERMANN METHOD

	Number of Cases.	+		—	
		+	%	—	+
Primary Syphilis .....	33	22=	66.6	8	3
Secondary Syphilis .....	120	104=	86.6	14	2
Tertiary Syphilis .....	91	66=	72.5	22	3
Early Latent Syphilis .....	81	39=	48.1	41	1
Late Latent Syphilis.....	74	33=	44.7	37	4
Hereditary Syphilis .....	4	4=	100	0	0
Cerebrospinal Syphilis .....	2	1=	50	1	0
Tabs .....	22	9=	40.9	13	0
Cerebral Endarteritis.....	2	2=	100	0	0
Suspected Syphilis .....	80	33=	41.2	39	8
Other Diseases .....	106	4*		102	0
	615	317		277	21

\* 1 Carcinoma, 1 Epithelioma, 1 Malaria, 1 Leprosy.

In Table 6 I present the results of the simultaneous examination of 244 cases of known syphilitic conditions by my own and by the Wassermann system. In my opinion, they prove conclusively the greater delicacy of my test over the other method in cases of syphilis. This greater percentage of positive reactions is not due to an undue sensitiveness of the method employed by me, but to the elimination in my system of the error introduced by using too great a quantity of amboceptor, as pointed out above.

TABLE 6.—COMPARISON OF THE RESULTS

	No. of Cases.	Wassermann Method.		Noguchi's Method.	
		+	%	+	%
Primary Syphilis .....	23	17=	73.9	6	20=
Secondary Syphilis .....	79	69=	87.3	10	76=
Tertiary Syphilis .....	65	52=	80	13	57=
Early Latent Syphilis .....	27	13=	48	14	18=
Late Latent Syphilis.....	32	24=	75	8	27=
Tabs .....	18	8=	44	10	13=
	244	183		61	211
					33

In conjunction with Dr. J. W. Moore, of the Manhattan State Hospital for the Insane, Ward's Island, New York, I have examined the cerebrospinal fluids of 200 cases of parasyphilitic and other nervous conditions; the results will appear in the July number of the *Journal of Experimental Medicine*.

175 West Seventy-second Street.

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## THE LEUCIN FRACTION OF PROTEINS.

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The present paper is intended, primarily as a contribution to the more exact methods for estimating the amino-acids from proteins, and, secondarily, to those for preparing l-leucin, d-isoleucin and d-valin from natural sources. Of the amino-acids obtained from proteins, tyrosin and glutaminic acid can be determined with a fair degree of accuracy, and the same is true of the three hexone bases and amide ammonia, as shown especially by the recent paper of Osborne, Leavenworth, and Brautlecht.<sup>1</sup> Of the thirteen remaining known amino-acids, a majority have been made readily accessible, and in better yields than previously possible by Fischer's well-known ester method. The far-reaching effects of Fischer's method, not only in the recent advance towards a more exact knowledge of protein chemistry, but in almost every field of biology, indicate the importance of developing quantitative accuracy in this field. The yields given by the original ester method were kept below quantitative by at least two sources of loss, the incompleteness with which the esters are extracted from the hydrolytic mixture, and the difficulties attending isolation of the separate acids from the groups in which they are obtained from the fractionated esters. The first source of loss is probably due either to incomplete esterification or to saponification of part of the esters before they can be extracted. It has been largely obviated by removing the mineral matter from the residues left after extracting the esters, and re-esterifying for a second and third extraction the amino-acids, usually nearly half the total, left in these residues. The first method used for this purpose was that of Abderhalden,<sup>2</sup> who changed by means of gaseous hydrochloric acid the masses of sodium hydrate and potash, used by

<sup>1</sup> *Amer. Journ. of Physiol.*, iii, p. 180, 1908.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, xxxvii, p. 484, 1903.

Fischer to free the esters, into chlorides, which were removed by repeated concentration of their alcoholic solution. A technique more convenient, and, we believe, less liable to loss has been developed in this laboratory.<sup>1</sup> The esters are freed for extraction from their hydrochlorides by means of barium hydrate, instead of sodium hydrate and potash, the barium being more easily handled and removed.

The esters are quite sharply separated by distillation into two chief fractions, one boiling above, the other below 90°, at less than 1 mm. pressure. The higher fraction contains glutaminic acid, phenylalanin, aspartic acid, and serin. With these the present paper is not concerned. We may simply mention that the determination of the first may be made quite accurately, of the second fairly so, while aspartic acid figures are less satisfactory, and serin not even roughly approximate. The lower boiling fraction contains the six acids, leucin, isoleucin, valin, prolin, alanin, glycocoll. Prolin can be separated from the others by means of its ready solubility in alcohol. This entire ester fraction is usually separated during distillation into at least two sub-fractions, the lower containing most of the alanin and glycocoll, the higher most of the valin and of the leucin isomers. All these acids are likely to be present in some proportion in each subfraction, however, so that the problem of their separation is unavoidable. The alanin and glycocoll, because of their ready solubility in water, can be separated from the three higher acids, the "leucin fraction," to a fair degree of completeness by fractional crystallization; then from each other by precipitating the glycocoll as ester hydrochloride, or more conveniently when, as usual, small amounts are present, as glycocoll picrate.<sup>2</sup>

The separation of leucin, isoleucin, and valin from one another with a completeness even approximate has been an unsolved problem. The three acids form isomorphous mixtures which are absolutely inseparable by crystallization, the only method in general use; and fractional crystallization of the copper salts is

<sup>1</sup> Levene: this *Journal*, i, p. 4, 1905; Levene and Alsberg: *Ibid.*, ii, p. 128, 1906; Levene and Van Slyke: *Biochem. Zeitschr.*, xiii, p. 442, 1908; for details of method cf. next paper, p. 419.

<sup>2</sup> Levene: this *Journal*, i, p. 413, 1906.

equally futile.<sup>1</sup> Consequently in the numerous hydrolyses published from Fischer's laboratory, especially by Abderhalden, the substances were not usually separated, the entire mixture being reported as "leucin."<sup>2</sup> That the proportion of valin and isoleucin, particularly of the former, in the leucin so reported may have been very large in many cases is indicated by our results in the next paper.

The most thorough and systematic work on the leucin fraction has been done by F. Ehrlich,<sup>3</sup> who discovered isoleucin, and with Wendel first isolated and determined the rotation of natural l-leucin. Ehrlich and Wendel employed the first systematic method for separating the three acids of this fraction. They were changed to copper salts, which were extracted with methyl alcohol. The isoleucin and valin salts dissolve, leaving the leucin in the residue. To separate the isoleucin from the valin it was necessary to free the acids from the copper and racemize the valin by heating the mixture of the two with barium hydrate solution in an autoclave. The copper salts were then regenerated and extracted with cold methyl alcohol or boiling 96 per cent ethyl alcohol, which removed the isoleucin salt and left the d-l-valin undissolved. This method requires lengthy manipulation, and gave only qualitative results. In the preliminary separation of leucin from valin and isoleucin the acids must be repeatedly regenerated and converted back into copper salts, which are again extracted with methyl alcohol in order to make the separation complete. The method also involves racemization of the d-valin, during which the d-isoleucin is partially

<sup>1</sup> Fischer: *Untersuchungen über Aminosäuren, Polypeptide und Proteine*, p. 642, 1906.

<sup>2</sup> Fischer: *loc. cit.* p. 67, "Aus der eben geschilderten Schwierigkeit, reines Leucin (besonders in der aktiven Form) aus dem Gemisch der Aminosäuren abzuscheiden, ergibt sich schon, dass von einer genauer quantitativen Bestimmung dieser Aminosäure nicht die Rede sein kann. Die Zahlen, die für Leucin in den zahlreichen, aus dem hiesigen Institut publizierten Hydrolysen von Proteinen angegeben sind, beziehen sich alle nicht auf das ganz reine Präparat, sondern vielmehr auf ein Gemisch mit Isoleucin, dem auch noch wechselnde Mengen von Aminovaleriansäure (Valin) beigemengt sein können."

<sup>3</sup> For references, cf. Ehrlich and Wendel: Zur Kenntnis der Leucinfraction des Eiweisses, *Biochem. Zeitschr.*, viii, p. 399, 1908.

changed to the isomeric d-allo-isoleucin. The racemization is almost certain to involve losses, and renders the method unavailable for preparation of either natural valin or d-isoleucin.

We have been able to effect a simple and quantitative separation of the two leucins from valin, the acids being regained without loss or racemization. The leucin isomers, when freed from valin, can be separated from each other without difficulty.

#### *Separation of Leucin and Isoleucin from Valin.*

In brief, leucin and isoleucin are precipitated as analytically pure  $\text{Pb}(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2$ , when  $\frac{M}{2}$  lead acetate, in quantity *slightly more than equivalent* to the leucin and isoleucin present, is added to the hot ammoniacal solution of the mixture. The valin is regained analytically pure from the filtrate. The proportions in which the two leucins are present are determined by the rotation of their mixture in 20 per cent hydrochloric acid; and they may be separated if desired, by extraction of their copper salts with methyl alcohol.

That the lead salt has not previously been used for the separation of leucin from valin seems more remarkable from the fact that it was employed by Strecker sixty years ago to first fix definitely the empirical formula and base-combining power of leucin.<sup>1</sup> This appears to have been overlooked by recent investigators. Levene<sup>2</sup> independently used precipitation with lead acetate to obtain pure leucin for analysis; and this method in a hydrolysis reported during the past year by us,<sup>3</sup> gave in one case, in which the proper conditions were struck largely by chance, a roughly quantitative separation of leucin from valin. It appeared that, under properly determined conditions, the method might afford means for more accurate analysis of the leucin fraction than has hitherto been possible. The present work was undertaken to fix these conditions.

It was found that:

(1) The lead compound precipitated is pure  $\text{Pb}(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2$ , and the lead determination affords a quick and accurate test of the purity of the substance.

<sup>1</sup> *Annalen*, lxxii, p. 89, 1849.

<sup>2</sup> *Biochem. Zeitschr.*, v, 36, 1907.

<sup>3</sup> Levene and Van Slyke: *Biochem. Zeitschr.*, xiii, p. 466, 1907.

(2) From a 10 per cent solution of natural leucin in dilute ammonia, about 80 per cent of the leucin is precipitated by the addition of an equivalent of lead acetate.

(3) Two or three equivalents of lead precipitate little or no more completely than slightly over 1 equivalent.

(4) The presence of 3 per cent or more of d-valin in the solution makes the precipitation of the leucin practically quantitative. Apparently the valin exerts a salting-out effect on the lead-leucin salt, a property as unexpected as it is favorable for the separation.

(5) Valin is partially precipitated by an equivalent of lead acetate. If, however, valin and leucin are present together in hot solution, and only a small excess of lead is added above the amount required to precipitate the leucin, the precipitate consists of the pure leucin salt. The leucin salt appears because of its greater insolubility to be first thrown out of solution, the valin beginning to precipitate only when the leucin is completely removed and an appreciable excess of lead has been added besides.

(6) Ninety per cent alcohol appears the best medium for washing the precipitate. It does not precipitate impurities from the adherent mother liquors, and the lead salt is not appreciably soluble in it. If the alcohol is followed by ether, the precipitate can be dried *in vacuo* over sulphuric acid for analysis within an hour.

Although the lead salt is precipitated from an ammoniacal solution containing an excess of lead and valin, pure dilute ammonia dissolves considerable amounts of the leucin from the precipitate. Cold water is a better washing liquid, but may dissolve 2 to 4 per cent of the precipitate.

In view of the above facts the following procedure is employed for the separation.

#### *Precipitation of Leucin as Lead Salt.*

The leucin-valin mixture is carefully analyzed and the amount of the leucins present calculated from the carbon content.

$$\frac{\text{Per cent C} - 51.24}{3.68} \times 100 = \text{percent of leucin isomers in the mixture.}$$

51.24 = per cent C in valin; 54.92 = per cent C in leucin; 3.68 = 54.92 - 51.24.

The acids are pulverized, in order to facilitate subsequent solu-

tion, and suspended in 7 parts of water in a flask. A portion of the flocculent substance remains floating on the surface, but this does not interfere with subsequent operations. The water is heated to the boiling point, the flask then removed from the flame, and 1.5 cc. of concentrated aqueous ammonia added for each gram of substance. The flask is stoppered and shaken gently to dissolve the acids. If solution of the last particles takes place with difficulty the stopper may be loosened and the flask heated gently, but when the substance has been thoroughly pulverized this is usually unnecessary. When solution is complete, a measured amount of 1.1 M lead acetate solution (sp. gr., 1.2540 at 20°), 4 cc. for each gram of leucin calculated, is run slowly into the hot solution from a pipette or burette. The liquid in the flask is thoroughly stirred during the addition, in order to prevent accumulation of an excess of lead acetate in any portion of the solution, and consequent precipitation of valin. After the lead has been added the flask is stoppered and chilled in cold water. Usually an hour or more was allowed after the liquid had been cooled for precipitation to become complete, although a shorter time may be sufficient. The solution is filtered with suction on a Buchner funnel, or a Gooch crucible if small amounts are determined. The precipitate is removed as completely as possible from the flask, and pressed into a compact mass in the funnel. After the greater part of the adherent mother liquor is removed by suction, the precipitate is washed with several small portions of 90 per cent alcohol, then with ether, and dried *in vacuo* over sulphuric acid to constant weight. The purity of the salt is confirmed by lead determination.

In case the ratio of leucin: valin is greater than 2:1, as indicated by a carbon content of the mixture exceeding 53.7 per cent the precipitation of the leucin will fall somewhat short of quantitative, because valin is not present in sufficient concentration to throw it out of solution. In such cases a smaller proportion of lead may be taken, 3.7 cc. per gram of calculated leucin (3.48 cc. = exact equivalent) and the filtrate from the lead-leucin concentrated *in vacuo* until the valin has reached a concentration of about 10 per cent. Ammonia is then added, precipitating the remainder of the lead-leucin, which is washed and filtered as described above. If it is preferred, however, the filtrate may be

treated at once, after the first crop of lead-leucin is obtained, by the method described in the following paragraph for obtaining valin from the filtrate. The valin obtained in this case will be accompanied by some leucin. It is consequently analyzed and submitted a second time to the lead precipitation, which invariably completes the separation.

#### *Recovery of Valin.*

The valin is recovered quantitatively from the lead-leucin filtrate by precipitating the excess of lead with hydrogen sulphide, and evaporating the filtrate from the lead sulphide to dryness on a water bath. The valin is taken up in 3:1 alcohol-ether mixture, and washed with it in order to remove adherent traces of acetic acid and ammonium acetate. A slight amount of valin dissolves, but is regained by evaporating the filtrate to dryness, and taking up the slight amount of residue left with the alcohol-ether as before.

#### *Purification of Lead-Leucin Salt.*

In case, for any reason, too great an excess of lead has been employed, resulting in contamination of the lead-leucin by lead-valin which will be indicated by the high lead content of the precipitate, the latter may be purified by dissolving and reprecipitating. It is pulverized and dissolved in 5 parts of hot water plus one-fourth part (1 equivalent) of glacial acetic acid. When solution is complete the lead-leucin is reprecipitated by adding 0.5 cc. of concentrated ammonia for each gram of the salt. The precipitate after cooling, is collected and washed as previously described. As the proportion of valin, estimated from the lead content of the original precipitate, is usually small, the last portions of leucin must be regained from the filtrate by concentration *in vacuo*, or repurifying the residue as described in the second paragraph above.

#### *Analysis of the Lead Salt.*

The determination of lead in the leucin salt furnishes a quick and accurate means for testing its purity. The analysis is conveniently performed as follows: The sample, about 0.3 gram, is dissolved in 5 cc. of  $\frac{N}{1}$  nitric acid in a 100 cc. beaker. No

heating is required. The lead is precipitated by addition of 5 cc. of  $\frac{N}{2}$  sulphuric acid, followed by 50 cc. of absolute alcohol. (If alcohol is added before the sulphuric acid the precipitate is so fine that filtration is difficult.) The lead sulphate is obtained in beautiful granular form, which settles rapidly and may be filtered in 15 minutes. The precipitate is collected in a Gooch crucible and washed with 95 per cent alcohol acidified with sulphuric acid. The Gooch is set into an ordinary porcelain crucible, heated gently until the alcohol is driven off, then with the full heat of the burner for ten minutes. Duplicates by this method usually agree within a few hundredths of a per cent.

#### *Properties of Lead-Leucin.*

The salt is precipitated in flocculent form, and is easily packed into a porous cake on the filter, so that the adherent liquid is removed by the suction. Owing to its physical character, it may be freed from impurities with a small amount of washing liquid. When cold water was used several small portions totaling a volume less than that of the solution in which the salt was precipitated were found sufficient. The salt is practically insoluble in 90 per cent alcohol, so that when this is used as washing liquid, the volume does not need to be kept so small, although, judging from the analytical purity of the precipitates obtained, the alcohol is as efficient as water in removing the mother liquors.

The lead-leucin when dry appears light and flocculent, like free leucin. It shows no evidence of water of crystallization, and is not hygroscopic. After being freshly precipitated and washed with water, it may be completely dried *in vacuo* over night at room temperature. When washed with alcohol and ether, as mentioned before, less than an hour is required for drying.

On ignition the salt yields, not lead oxide but metallic lead in shining globules. If the ignition is performed carefully, and stopped as soon as the organic matter is removed, a yield of metallic lead only slightly above the theoretical is obtained. Repeated treatments in nitric acid and re-ignition are required to change the lead completely to lead oxide, so that analysis by lead oxide determination is not satisfactory. When the substance is burned a trace of carbon is apparently enclosed by the lead as carbon determinations usually come out several tenths below theoretical,

though performed on substances which determinations of Pb, N, and H show to be analytically pure. The sulphate method detailed above, however, leaves little to be desired in convenience or accuracy.

The leucin salt is readily soluble in dilute nitric and acetic acids, soluble slowly in about 100 parts of distilled water at room temperature.

#### *Regeneration of Leucin from the Lead Salt.*

In order to regain the free leucin quantitatively, the lead salt is dissolved in 15 to 20 parts hot water plus one-fourth part of glacial acetic acid, and freed from lead with hydrogen sulphide. The precipitate is filtered on a Buchner funnel, the filter paper being re-enforced by a thin mat of asbestos fiber. The lead sulphide is washed thoroughly with hot water. The filtrate is evaporated to dryness on a water bath or *in vacuo*, and the leucin washed with a small amount of 1:1 absolute alcohol-ether mixture to remove traces of acetic acid.

#### *Polarimetric Determination of Leucin and Isoleucin.*

Ehrlich has shown that l-leucin is not racemicized by heating with acids, and that it is obtained with full optical activity from proteins after hydrolysis with acids. We have found the same to be true of d-isoleucin as will be shown later. Consequently when the two are obtained quantitatively in analytically pure condition their proportions may be calculated from the specific rotation of the mixture. Because of the decided difference ( $21.8^\circ$ ) between the rotations of the two in 20 per cent hydrochloric acid, this method is probably more accurate than any direct separation, and is, of course, easily executed. It is found that the rotations of the isomers are strictly additive, neither affecting by its presence the rotation of the other; and consequently a simple calculation gives the composition of a mixture of known optical activity. Ehrlich has established the specific rotation of d-isoleucin in 20 per cent hydrochloric acid as  $+36.80$ . We find however, that d-isoleucin obtained from proteins and purified by methods more effective than those available to Ehrlich has the slightly higher rotation  $+37.4^\circ$ , and employ this figure. Fischer and Warburg

have found a rotation of  $+15.6^\circ$  for l-leucin from the brucin salt of synthetic formyl leucin;<sup>1</sup> and Ehrlich and Wendel<sup>2</sup> have found the same value for l-leucin from hydrolyzed proteins. Using these values,<sup>3</sup> the composition of a mixture of specific rotation  $R$  is calculated by the formulæ:

$$\text{Per cent d-isoleucin} = 100 \times \frac{R - 15.6}{21.8}$$

$$\text{Per cent l-leucin} = 100 \times \frac{37.4 - R}{\phantom{21.8}}$$

### *Separation of Leucin from Isoleucin.*

The isomers, free from valin and other impurities, can be separated from each other by extraction of their copper salts with methyl alcohol, as shown by F. Ehrlich for the leucins from beet sugar residues, and by Levene and Jacobs<sup>4</sup> for those obtained from hydrolyzed casein by lead precipitation. Consequently the method above detailed affords more accessible means than have previously been known for the preparation in quantity of natural valin and d-isoleucin, and simplifies that of l-leucin. A kilo of casein contains at least 87 grams of l-leucin, 67 grams of valin, and 15 grams of d-isoleucin, as we have determined by the methods outlined.

The isolation of d-isoleucin has previously been interfered with by the presence of d-valin, because the copper salts of both have nearly the same solubilities in methyl alcohol (1:55 and 1:52 respectively at room temperature), and the mixture of the two obtained when Ehrlich's method is applied to the leucin fraction is inseparable even qualitatively by former methods. This has prevented the preparation of more than minute amounts of d-isoleucin from proteins. Ehrlich's source has been the concentrated

<sup>1</sup> *Ber. d. deutsch. chem. Gesellsch.* xxxviii, p. 3497, 1905.

<sup>2</sup> *Loc. cit.*

<sup>3</sup> Locquin (*Bul. soc. chim. d. France*, (4), i, p. 601, 1907) reports a specific rotation of  $+40.6^\circ$  for d-isoleucin obtained from the brucin salt of the formyl derivative of synthetic d-l-isoleucin. Ehrlich's figure  $+36.8^\circ$ , which is generally accepted, agrees more closely with our own,  $+37.4^\circ$ . We hope to prepare a larger amount of pure material, and settle the question concerning the exact rotation.

<sup>4</sup> *Biochem. Zeitschr.*, p. 231, ix, 1908.

residue from beet sugar molasses, from which a mixture of the leucin isomers free from valin was obtained in yields of 1 to 2 grams per kilo.

#### EXPERIMENTAL.

##### *Separation of Leucin and Isoleucin from Valin.*

The following experiments demonstrate the precipitability, as lead salt, of leucin in pure solution; the effect of valin upon the precipitability of leucin; and the completeness with which valin can be separated from the leucins by the lead method. The leucin used showed a specific rotation in 20 per cent hydrochloric acid of  $+17.2^\circ$ , the valin of  $+27.4^\circ$ . The leucin gave, on analysis, 54.80 per cent C, 10.07 per cent H, instead of the theoretical 54.92 per cent C. and 9.99 per cent H. From its rotation it contains 92.5 per cent l-leucin, 7.5 per cent d-isoleucin. The valin gave 51.40 per cent C and 9.67 per cent H, instead of 51.24 per cent C and 9.47 per cent H. Both were obtained from hydrolysis of casein.

Nos. 1 and 2. Two grams of leucin dissolved in 14 cc. of water + 3 cc. aqueous ammonia, and precipitated by 7.5 cc. of 1.16 M lead acetate (14 per cent excess).

Nos. 3 and 4. Two grams of leucin plus 2 grams of valin dissolved in 24 cc. of water plus 5 cc. ammonia and precipitated by 7.5 cc. of 1.16 M lead acetate.

The solutions were all cooled in ice water, filtered in Gooch crucibles, the precipitates washed with small amounts of ice water, alcohol not having been tested previously, and dried to constant weight *in vacuo*. The theoretical amount of lead-leucin from 2 grams of leucin is 3.563 grams. The following amounts were obtained:

No.....	1	2	3	4
Amount Pb-leucin. . .	2.724	2.892	3.528	3.567
Per cent precipitated ..	76.45	81.17	98.95	99.65

The precipitates gave the following figures on analysis:

No.....	1	2	3	4
Substance.....	0.3552	0.2984	0.3377	0.3822
PbSO <sub>4</sub> .....	0.2298	0.1931	0.2197	0.2485
Per cent Pb.....	44.18	44.19	44.43	44.45

Per cent Pb calculated for Pb (C<sub>6</sub>H<sub>12</sub>O<sub>2</sub>N)<sub>2</sub>, 44.29.

The leucin from precipitates 3 and 4 was quantitatively regenerated, as previously described, and gave without purification, the following figures on analysis:

0.1452 gram substance; 0.2916 gram  $\text{CO}_2$ ; 0.1298 gram  $\text{H}_2\text{O}$

	Calculated for $\text{C}_6\text{H}_{13}\text{O}_2\text{N}$ :	Found:
C .....	54.92 per cent.	54.77 per cent.
H .....	9.99 per cent.	9.99 per cent.

The filtrates from the lead precipitates of 3 and 4 gave 1.919 and 1.920 grams of valin respectively, or 96 per cent each of the original 2 grams. As the substance was collected in Buchner funnels a slight loss was unavoidable in removing it from them. The samples were analyzed as follows:

No.....	3	4
Substance.....	0.1448	0.1528
$\text{CO}_2$ .....	0.2732	0.2888
$\text{H}_2\text{O}$ .....	0.1239	0.1325
Per cent C.....	51.45	51.37
Per cent H.....	9.53	9.70
Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ ; 51.24 per cent C; 9.47 per cent H.		

The following experiments were performed to further test the effect of the presence of valin upon the precipitability of the leucins. The same valin was employed, but a different leucin, of specific rotation  $+25.12^\circ$  in 20 per cent hydrochloric acid, and, from this, containing 44.8 per cent isoleucin, 54.2 per cent leucin. It gave on analysis 55.11 per cent C, 10.02 per cent H. In each case the amino acids were dissolved in 12 cc. of water plus 2 cc. of ammonia, and precipitated with 3.75 cc. of 1.16 M lead acetate (0.45 cc. or 14 per cent above the equivalent of 1 gram of leucin). The precipitates were washed with 90 per cent alcohol. The theoretical yield is 1.781 gram Pb-leucin.

No.....	1	2	3	4
Leucins.....	1.000	1.000	1.000	1.000
Valin.....	None	0.250	0.500	1.000
Pb-leucin.....	1.404	1.704	1.834	1.829
Per cent leucin pre- cipitated.....	78.83	95.68	103.0	102.7

## ANALYSES

Substance.....	0.3257	0.3054	0.3410	0.3164
PbSO <sub>4</sub> .....	0.2111	0.1985	0.2209	0.2054
Per cent Pb.....	44.26	44.39	44.34	44.33

Calculated for Pb (C<sub>6</sub>H<sub>12</sub>O<sub>2</sub>N)<sub>2</sub>; 44.29 per cent Pb.

The precipitates were combined and the leucin regenerated.

Analyses: 0.1319 gm. substance; 0.2649 gm. CO<sub>2</sub>; 0.1162 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>12</sub> O <sub>2</sub> N:	Found:
C.....	54.92 per cent.	54.78 per cent.
H.....	9.99 "	9.86 "

The marked effect of even a small amount of valin is seen in the difference between Nos. 1 and 2. In No. 3 the maximum of leucin is precipitated. Here 0.500 gram of valin was dissolved in about 18.cc. of liquid, making approximately a 2.8 per cent solution of valin. Evidently leucin is precipitated completely from a 1:40 solution of valin. The fact that 3 per cent more than the theoretical amount of lead-leucin was obtained may be due to either a slight amount of lead-valin in the precipitate, or to the presence of a small amount of leucin in the valin used. The presence of so small a proportion of either acid as impurity in the other could hardly be detected with certainty by analysis, as it would make a difference of only 0.08 per cent in the Pb content, or, 0.11 per cent in the carbon of the free acid.

*Solubility of Lead-Leucin.*

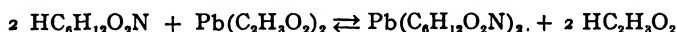
The following experiments indicate roughly the relative solubilities of the salt in alcohol, cold water, and dilute ammonia (1-10). Two precipitates of 1.5245 and 1.6143 grams weight in Gooch crucibles were washed slowly with 25 cc. each of 96 per cent alcohol, followed by a few cc. of ether. They lost 0.002 and 0.004 gram respectively. After similar washing with distilled water at about 10°, followed by a few cc. of alcohol and ether, the losses were .110 and .098 gram. When the experiment was repeated using dilute ammonia (1 cc. of concentrated ammonia to 10 cc. water) the losses were 0.183 and 0.164 gram.

The solvent action of water and dilute ammonia was determined in the following experiments. In each case 0.500 gram of lead leucin was shaken 16 hours with 25 cc. of liquid, and the

organic nitrogen determined in 20 cc. of the filtrate. For this purpose the ammonia was driven off from the ammoniacal solution by adding a few cc. of 50 per cent sodium hydroxide, diluting, and boiling in Kjeldahl flasks. The solutions were then digested as usual with sulphuric acid. The method was checked by controls, in which the organic nitrogen in both filtrate and precipitate was determined, and found accurate. The duplicates represent separate solutions, not duplicate Kjeldahls on the same solutions.

CC. H <sub>2</sub> O.	CC. CONC. NH <sub>4</sub> OH.	ORGANIC N IN 20 CC.		GRAMS LEUCIN DISSOLVED PER 100 CC.	
		1	2	1	2
25	none	0.0124	0.0125	0.578	0.583
23.75	1.25	.....	0.0232	.....	1.085
22.50	2.50	0.0233	0.0236	1.089	1.105
20.00	5.00	0.0228	0.0226	1.064	1.056

These results indicate that the lead-leucin is about half as soluble in water as in dilute ammonia. Apparently the ideal condition for precipitation would involve having only enough ammonia present to neutralize the acetic acid formed by the reaction,



which would otherwise prevent the precipitation of the lead leucin. The presence of valin and a slight excess of lead acetate in solution reduce the solubility of the leucin salt sufficiently, however, to make this precaution appear unnecessary.

#### *Precipitability of Other Amino Acids by Lead and Ammonia.*

Three-tenths gram of each acid were dissolved in 2 cc. of hot water (except tyrosin which required several cubic centimeters) plus 0.5 cc. of concentrated ammonia, then 1 cc. of 1.16 M lead acetate added. l-Tyrosin, l-phenylalanin, d-l-phenylalanin, d-valin, and d-l-valin were precipitated at once. d-Glutaminic acid precipitated on cooling. d-l-Aspartic acid precipitated only after a second cubic centimeter of lead acetate, making nearly an

equivalent, had been added. d-Alanin, l-prolin, l-oxyprolin, and d-l-serin were not precipitable.

Tyrosin when present with leucin, is precipitated with the latter as lead salt, so that the lead method for obtaining leucin cannot be used in the presence of tyrosin.

*Polarimetric Determination of Proportions of Leucin and Isoleucin in Mixture.*

Ehrlich's rotations of leucin in 20 per cent hydrochloric acid have all been taken with solutions of about 3.65 per cent concentration, while the rotation of isoleucin has been uniformly taken with solutions of about 4.5 per cent strength. In order to make certain that the polarimetric method is applicable for quantitative determinations, the effect of concentration upon the specific rotation must be known.<sup>1</sup> The following determinations, upon a sample of analytically pure leucin obtained by the ester and lead methods from casein, show that the specific rotation of leucin is within ordinary limits, independent of the concentration. The observations were all taken with a tube of 1.894 dm. length, and the specific gravity of the solutions was 1.099, not being appreciably affected by this change in leucin concentration.

NO.	WT. LEUCIN.	TOTAL WT. SOLUTION.	PER CENT LEUCIN.	OBSERVED ROTATION.	$[\alpha]_D^{20^\circ}$
				<i>degrees.</i>	<i>degrees.</i>
1.....	0.4201	18.225	2.304	+ .83	+17.29
2.....	0.5871	19.744	2.974	+1.04	+17.31
3.....	0.7689	22.698	3.386	+1.19	+16.87
4.....	0.6984	18.994	3.677	+1.32	+17.23
5.....	0.8808	19.140	4.603	+1.64	+17.10
6.....	1.0518	18.172	5.789	+2.09	+17.33

It is evident that, at least below 5.8 percent concentration, the specific rotation of leucin is practically independent of the con-

<sup>1</sup> W. Jones (this *Journal*, v, p. 1, 1908) has shown that the specific rotation of the nucleic acids varies markedly with the concentration. This is also true of some other substance (Vaubel, *Physik. u. chem. Methoden der quant. Best. org. Verbindungen*, i, p. 435.)

centration. The rotation of isoleucin is also constant as will be shown presently.

It remains to determine the mutual effect of leucin and isoleucin upon their rotations when in solution together. The following table shows that each exhibits its normal rotation unaffected by the other, the rotations of mixtures being additive. Neither the leucin nor the isoleucin used was entirely free from the other, but this is not necessary in order to demonstrate the simple additivity of their rotations. The leucin (A) was from the lot used in the preceding experiment. The isoleucin (B) also was analytically pure, and showed a rotation of  $33.46^\circ$  instead of  $36.80^\circ$ .

NO.	GRAMS A.	GRAMS B.	CONCENTRATION OF SOLUTION PER CENT	$[\alpha]_D^{20}$ OBSERVED.	$[\alpha]_D^{20}$ CALCULATED FROM AMT. A AND B.
1	Av. 6 rotations	.0000	2.3-5.8	+17.19	.....
2.	.7747	.2179	4.868	+20.96	+20.75
3...	.3870	.3870	4.051	+25.49	+25.35
4..	.2041	.5983	4.200	+29.10	+29.36
5 .	.0000	.8298	4.332	+33.46	.. .

The calculated and observed rotations agree within the limit of error. The calculated rotations are computed by the formula,

$$[\alpha]_D^{20} = 17.19 + \frac{a}{a+b} \times 16.37$$

$a$  representing the grams of A (leucin) in second column,  $b$  the grams of B (isoleucin) in the third.  $16.37^\circ = 33.46^\circ - 17.19^\circ$ , the difference between the rotations of A and B. The degree of accuracy of the polarimetric method is indicated by the following figures from the preceding data.

NO.	PER CENT A PRESENT.	PER CENT B PRESENT.	PER CENT A CALCULATED FROM ROTATION.	PER CENT B CALCULATED FROM ROTATION.
2.....	78.0	22.0	76.9	23.1
3.....	50.0	50.0	49.1	50.9
4.....	25.4	74.6	27.0	73.0

The calculated percentages are by the equation:

$$\text{Per cent B} = \frac{[\alpha]_D^{20} - 17.2}{16.37} \times 100.$$

$$\text{Per cent A} = 100 - \text{per cent B}.$$

From the above results it is evident that the rotation of a leucin-isoleucin solution is a simple linear function of the percentages of the constituents. Consequently, the percentages of the latter in a mixture of ascertained specific rotation in 20 per cent hydrochloric acid may be calculated by the equations already given.

The applicability of the above polarimetric method rests, of course, on the correctness of the assumption that l-leucin and d-isoleucin are the only leucin isomers occurring in natural proteins, and that these are not racemicized by acid hydrolysis. All the work which has been done on the natural amino-acids failed to disclose any indication of the presence of other isomers, and F. Ehrlich's<sup>1</sup> work on this point especially indicates the non-existence of other isomers in proteins. Ehrlich and Wendel<sup>2</sup> have also demonstrated conclusively that the l-leucin obtained by acid hydrolysis possesses full optical activity, and that this is not decreased in the slightest by 20 hours boiling of the pure l-leucin in 33 per cent sulphuric or concentrated hydrochloric acids. We have found the same to be true of d-isoleucin.

This was already rendered probable by the work of Weitzenboeck<sup>3</sup> and of Ehrlich and Wendel. Weitzenboeck obtained from casein by acid hydrolysis and esterification, isoleucin of  $[\alpha]_D^{20} = +38.3^\circ$  in 22 per cent hydrochloric acid. The deviation from Ehrlich's figure is about what would be expected from the difference in the concentration of hydrochloric acid used as solvent. Loquin<sup>4</sup> found that changing the concentration of the solvent hydrochloric acid solution from 8 per cent to 12 per cent increased the specific rotation of isoleucin by  $9^\circ$ , an average of  $\frac{3}{4}^\circ$  for each per cent increase in hydrochloric acid. Ehrlich and Wendel obtained mixtures of isoleucin and valin from acid hydrolyses

<sup>1</sup> *Ber. d. deutsch. chem. Gesellsch.* xxxvii, p. 1809, 1909.

<sup>2</sup> *Biochem. Zeitschr.*, viii, p. 412, 1908.

<sup>3</sup> *Monatsh. f. Chem.*, xxvii, p. 831, 1906.

<sup>4</sup> *Bull. soc. chim. d. France*, (4), i, p. 160, 1907.

of casein, spongin and ovalbumin, the rotations of which varied between  $+30.00^\circ$  and  $+32.40^\circ$ , the carbon from 51.77 per cent to 53.76 per cent, nitrogen accordingly. The rotation of d-valin is  $+28.8^\circ$ , of d-isoleucin  $+37.4^\circ$ , the corresponding carbon contents being 51.24 per cent and 54.96 per cent. It is evident that the isoleucin could have lost little of its optical activity, or its presence would not have so markedly raised the rotation of the mixture above that of d-valin.

We have found that isoleucin is obtained in full optical activity from casein hydrolyzed with acid and subjected to the usual ester method; and that its rotation is not affected by 18 hours boiling with 20 per cent hydrochloric acid. As a preliminary experiment, a 1.3 gram portion of isoleucin previously prepared by Levene<sup>1</sup> of rotation  $34.6^\circ \pm 0.3^\circ$ , was boiled 18 hours with 20 per cent hydrochloric acid. The solution was then freed from hydrochloric acid by means of silver sulphate followed by hydrogen sulphide and the required amount of barium hydrate. 1.14 grams of isoleucin were regained by concentrating the solution almost to dryness, and crystallizing from alcohol.

Rotation in 20 per cent hydrochloric acid: 0.7280 gram substance; 19.212 grams total solution; per cent isoleucin, 3.790; rotation in 1.894 dm. tube,  $+2.76^\circ$ ; sp. gr. 1.099.

$$[\alpha]_D^{20} = +34.96^\circ \pm 0.3^\circ$$

In order to test the effect of concentration on the specific rotation, 9.922 grams of the above solution were diluted to 19.180 grams with 20 per cent hydrochloric acid, making a 1.96 per cent solution of isoleucin. This showed a rotation of  $+1.43^\circ$  in a 1.894 dm. tube.

$$[\alpha]_D^{20} = +35.0^\circ \pm 0.5^\circ$$

It appears that boiling in strong acid, such as is used for hydrolysis, does not affect the rotation of isoleucin, and that the specific rotation is, like that of l-leucin, independent of the concentration, within ordinary limits.

#### *Preparation of Pure d-Isoleucin from Casein.*

The isoleucin used for the above experiments evidently contained a small amount of l-leucin, its rotation being  $2^\circ$  below

<sup>1</sup> Levene and Jacobs: *Biochem. Zeitschr.*, ix, p. 231, 1908.

that given by Ehrlich for d-isoleucin. In order to make the test more rigid another portion of the substance was prepared, with full optical activity according to Ehrlich's figures. The source was 10 grams of leucin obtained from casein hydrolyzed with 25 per cent hydrochloric acid. The leucin was obtained by the previously described lead method from the esters boiling between  $60^{\circ}$  and  $80^{\circ}$  at 0.5 mm. It showed a specific rotation of  $+22.3^{\circ}$  in 20 per cent hydrochloric acid indicating 31 per cent of d-isoleucin, 69 per cent of l-leucin. The substance was transformed into copper salts by boiling with a small excess of copper oxide, and extracting the latter with large volumes of boiling water to remove the difficultly soluble leucin salt. The solution was concentrated to dryness *in vacuo*. The dry copper salts were ground fine and extracted 48 hours in a shaking machine with 500 cc. of 94 per cent Merck's methyl alcohol, then washed thoroughly with the same solvent. Apparently the l-leucin salt is slightly soluble, for shaking with successive portions of methyl alcohol continued dissolving slight amounts of copper salt; although the first extraction was performed with enough alcohol to take up 9 grams of isoleucin-copper. The first extraction yielded 2.9 grams of copper salt, and only this was used for isoleucin. The copper content was determined by Volhard titration.

0.1715 gram of substance required 5.40 cc. of  $\frac{N}{10}$   $\text{NH}_4\text{SCN}$ , indicating 0.0343 gram Cu or 20.02 per cent, the theoretical being 19.64 per cent Cu. The salt was decomposed with hydrogen sulphide, and the isoleucin purified by precipitating once as lead salt, then recrystallizing from dilute alcohol.

Analysis: 0.1535 gram substance; 0.3090 gram  $\text{CO}_2$ ; 0.1409 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_8\text{H}_{18}\text{O}_2\text{N}$ :	Found:
C. ....	54.92 per cent	59.90 per cent.
H. ....	9.99 "	10.27 "

Rotation in 20 per cent hydrochloric acid: 0.6116 gram substance 18.968 grams solution; rotation in 1.894 dm. tube,  $+2.40^{\circ}$ ; sp. gr. 1.099.

$$[\alpha]_D^{20} = +35.76^{\circ}.$$

As noted above, it appeared that a slight amount of l-leucin copper might have been dissolved, even at room temperature, by the large excess of methyl alcohol used. Consequently the isoleucin was changed back into the copper salt, which was extracted

with only 150 cc. of methyl alcohol. A portion remained undissolved. The soluble portion yielded isoleucin of the following rotation.

0.5158 gram substance; 11.372 grams total solution; concentration of solution, 4.536 per cent; sp. gr., 1.099; rotation in 0.865 dm. tube,  $+1.61^{\circ} \pm .01^{\circ}$

$$[\alpha]_D^{20} = +37.35^{\circ} \pm 0.23^{\circ}$$

5.89 grams of the above solution were diluted to 11.427 grams with 20 per cent hydrochloric acid making a 2.34 per cent solution. Rotation in 0.865 dm. tube,  $+0.825^{\circ} \pm .01^{\circ}$

$$[\alpha]_D^{20} = +37.10^{\circ} \pm 0.4^{\circ}$$

The isoleucin was boiled 18 hours with 20 per cent hydrochloric acid freed from hydrochloric acid as described before, and its rotation in 20 per cent hydrochloric acid repeated.

0.2224 grams substance; 11.200 grams solution; concentration of solution 1.986 per cent; sp. gr., 1.099; rotation in 0.865 dm. tube,  $+0.696^{\circ} \pm .01^{\circ}$

$$[\alpha]_D^{20} = +36.9^{\circ} \pm 0.5^{\circ}$$

The above results indicate:

- (1) That d-isoleucin is not racemicized by prolonged boiling with strong hydrochloric acid.
- (2) That it is obtained with full optical activity from proteins hydrolyzed with acid and subjected to the ester method.
- (3) That the specific rotation is the same at different concentrations.

Consequently, the same properties having been demonstrated for l-leucin, the polarimetric method may be relied upon for the determination of l-leucin and d-isoleucin obtained by acid hydrolysis of proteins.

#### *d-Isoleucin from Edestin.*

The amino acids of the leucin fraction obtained, after acid hydrolysis of edestin, partly by crystallization, partly by the ester method, were changed to copper salts, and the latter extracted with methyl alcohol. From the copper salts soluble in methyl alcohol, 13 grams of crude isoleucin-valin mixture were regained. As the substance was considerably tinged with brown, it was not subjected to preliminary analysis, but at once dissolved and precipitated with lead-equivalent to 8 grams of isoleucin.

The filtrate from the lead-isoleucin yielded 6 grams of pure recrystallized valin, giving 51.47 per cent C, 9.21 per cent H on analysis, and showing specific rotation of  $+26.8^\circ$  in 20 per cent hydrochloric acid.

The lead salt was decomposed, and yielded 3.6 grams of isoleucin, which, because of the large excess of lead that had been used, still contained a small amount of valin.

Analysis: 0.1226 gram substance; 0.2440 gram  $\text{CO}_2$ ; 0.1111 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{13}\text{O}_2\text{N}$ :	Found:
C.....	54.92 per cent	54.28 per cent.
H.....	9.99 "	10.14 "

The specific rotation in 20 per cent hydrochloric acid was  $+35.3^\circ$ , that found by us for purified natural isoleucin from casein being  $+37.35^\circ$ .

To free entirely from valin, 3.2 grams of the substance were dissolved as usual and precipitated with an equivalent of lead acetate. 2.5 grams of isoleucin were regained, of the following rotation:

0.3024 gram substance; 18.731 gram solution; concentration, 1.618 per cent; sp. gr., 1.099; rotation in 1.855 dm. tube,  $+1.19^\circ$ .

$$[\alpha]_D^{20} = +36.91^\circ \pm 0.3^\circ$$

To complete the purification, the isoleucin was transformed again into the copper salt, 2.56 grams of which were shaken over night with 170 cc. of methyl alcohol. This was only slightly more than the amount required to dissolve the copper salt of pure isoleucin, and insufficient to take up appreciable amounts of l-leucin copper salt. All but 110 mg. of the copper salt dissolved in the methyl alcohol. The solution was concentrated to dryness, and the salt dissolved in water and decomposed with hydrogen sulphide. The isoleucin regained was passed once more through the lead process, and crystallized from a small amount of alcohol. 1.47 grams were regained.

Rotation in 20 per cent hydrochloric acid; 0.6978 gram substance; 18.732 gram solution; concentration, 3.726 per cent; sp. gr., 1.099; rotation in 1.855

dm. tube, + 2.85° and + 2.84° respectively by two observers, average, + 2.845°

$$[\alpha]_D^{20} = + 37.44^\circ \pm 0.15^\circ$$

Analysis: substance, 0.1430 gram; CO<sub>2</sub>, 0.2876 gram; H<sub>2</sub>O, 0.1269 gram.

	Calculated for C <sub>9</sub> H <sub>18</sub> O <sub>2</sub> N:	Found:
C.....	54.92 per cent.	54.86 per cent.
H.....	9.99       "	9.93       "

The fact that careful purification, through the copper and lead salts and recrystallizing, raised the rotation only 0.5° indicates that the product is about as pure as can be obtained from natural sources by present methods. The rotation is identical with that (+ 37.35°) of the isoleucin from casein.

#### *Separation of Leucin and Valin as Obtained from Casein.*

Following is a description of the manipulation of a fraction of amino acids which consisted mainly of the equimolecular mixture of leucin and valin which defies separation by the fractional recrystallization method. The fraction was obtained from the third crop of amino acids crystallizing from several pounds of casein which had been digested six weeks with trypsin, in order to prepare amino-acids for physiological experiments. The crop of crystals referred to was esterified, the esters freed with barium hydrate, and distilled, yielding the following fractions:

Fraction	Temp. vapors. degrees.	Pressure. mm.	Weight of esters. grams.
I .....	to 70°	15	28
II .....	60°-80°	0.25	111
III .....	80°-120°	0.40	63

Fraction II contained chiefly the leucin fraction. It was separated into subfractions, with the object of obtaining mixtures of the leucins and valin in variations covering all the range likely to be met with in proteolytic products. We have shown previously that extraction of the mixed esters with ether gives an ether-soluble portion containing most of the leucin, and a water-soluble

containing most of the valin.<sup>1</sup> This method was combined with fractional crystallization.

The esters were poured into 3 volumes of water, and the mixture extracted with an equal volume of ether. The ethereal solution was washed six times with water, a volume of the latter equal to about two-thirds that of the ethereal solution being used for each washing. The esters contained in the water and ether respectively were saponified separately by boiling with water, and the amino-acids from the former separated into four, those from the latter into two fractions by crystallization. The leucin and valin in each were separated by the lead method. Fractions 5 and 6 were freed from prolin by boiling with alcohol. It is noteworthy that the ether-soluble esters were entirely free from prolin.

The fractions were of the following quantity and analysis:

NO.	WEIGHT IN GRAMS.	PER CENT C.	PER CENT H.	REMARKS.
1.....	37.43	54.26	9.96	From ether soluble esters
2.....	8.32	52.74	9.27	" " " "
3.....	8.13	53.74	10.09	" water " "
4.....	12.54	53.52	9.55	" " " "
5.....	7.50	52.79	9.39	" " " "
6.....	0.48	47.13	8.55	" " " "

*Fraction 1.* 37.43 grams, 54.26 per cent C. From the carbon content, this fraction should contain 81.5 per cent or 30.5 grams of leucin. Because of its high proportion, the leucin had to be removed in two portions (cf. p. 396). The mixture was dissolved in 250 cc. hot water and 50 cc. concentrated ammonia, and precipitated with 120 cc.  $\frac{M}{10}$  lead acetate, equivalent to 31.4 grams leucin. 51.43 grams of lead-leucin were precipitated, equivalent to 28.87 grams of leucin. Analysis:

- I. 0.3705 gm. substance, 0.2397 gm.  $\text{PbSO}_4$
- II. 0.2313 gm. " 0.2558 gm.  $\text{CO}_2$ , 0.1050 gm.  $\text{H}_2\text{O}$ .
- III. (1) 0.4330 gm. " 18.45 cc.  $\frac{N}{10}$   $\text{H}_2\text{SO}_4$  (Kjeldahl)
- (2) 0.4326 gm. " 18.28 cc.  $\frac{N}{10}$  " ( " )

<sup>1</sup> Levene and Van Slyke; *Biochem. Zeitschr.*, xiii, p. 442, 1908.

## The Leucin Fraction of Proteins

	Calculated for $\text{Pb}(\text{C}_6\text{H}_{13}\text{O}_2\text{N})_2$	Found:
Pb.....	44.29 per cent.	44.20 per cent.
C.....	30.83 "	30.14* "
H.....	5.18 "	5.07 "
N.....	6.00 "	$\left\{ \begin{array}{l} 5.98 \\ 6.03 \end{array} \right.$ "

\* Carbon as a rule is found low, probably because carbon particles are enclosed in the lead-globule formed when the salt decomposes.

Twenty grams of the salt were freed from lead, and the leucin regenerated. 10.77 grams were obtained, calculated 11.22 grams. Analysis: 0.1566 gram substance; 0.3164 gram  $\text{CO}_2$ ; 0.1404 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{13}\text{O}_2\text{N}$ :	Found:
C .....	54.92 per cent.	55.11 per cent.
H .....	9.99 "	10.02 "

Rotation in 20 per cent hydrochloric acid: 0.5072 gram substance; 11.125 grams total solution; sp. gr., 1.10; observed rotation in 0.865 dm. tube, + 1.09°.

$$[\alpha]_D^{20} = + 25.12^\circ$$

According to the rotation the "leucin" was at least 44.8 per cent isoleucin. As the substance was obtained from prolonged tryptic digestion in alkali, some racemization may have occurred, and the correct figure be somewhat higher.

The filtrate from the lead-leucin, freed from lead, evaporated to dryness and washed with absolute alcohol yielded 7.83 grams of a mixture of leucin and valin. The loss of 0.73 gram was not noticed until the wash alcohol had been discarded. Thereafter the wash alcohol was evaporated to dryness, and a second crop obtained, which made the recovery of the amino-acids quantitative.

The mixture gave on analysis 52.94 per cent C, 0.44 per cent H. From the carbon content the 7.83 grams should contain 46 per cent leucin or 3.60 grams, and 4.23 grams of valin. 7.64 grams were dissolved in 50 cc. of water plus 5 cc. ammonia, and precipitated with 15 cc.  $\frac{M}{7}$  lead acetate. 5.84 grams of lead-leucin, equivalent to 3.28 grams of leucin were obtained.

Analysis: (1) 0.3105 gm. substance; 0.2018 gm.  $\text{PbSO}_4$ ; 44.38 per cent Pb.  
(2) 0.3413 " " ; 0.2214 " " ; 44.30 " "  
Calculated for  $\text{Pb}(\text{C}_6\text{H}_{13}\text{O}_2\text{N})_2$ : 44.29 per cent Pb.

The filtrate yielded 4.20 grams of valin.

Analysis: 0.1523 gram substance; 0.2859 gram CO<sub>2</sub>; 0.1281 gram H<sub>2</sub>O.

	- Calculated for C <sub>5</sub> H <sub>11</sub> O <sub>2</sub> N:	Found:
C.....	51.24 per cent.	51.21 per cent.
H.....	9.47 "	9.41 "

Rotation of valin in 20 per cent hydrochloric acid: 0.4730 gram substance; 11.220 gram total solution; sp. gr., 1.10; obtained rotation in 0.865 dm. tube, + 1.02°.

$$[\alpha]_D^{20} = + 25.4^{\circ}.$$

The rotation agrees with that found by previous observers for natural valin, although lower than the synthetic d-valin of Fischer, which showed a specific rotation of + 28.8° ± 0.4°.<sup>1</sup> The rotation of valin from natural sources varies through a range of several degrees below this, presumably because it is somewhat racemized. The possibility is not excluded, of course, that the variations may be due to the presence of small amounts of the low-rotating isovalin synthesized by Ehrlich, as the high rotation formerly attributed to leucin was due to the presence of small amounts of high-rotating isoleucin.<sup>2</sup>

The yields of leucin and valin calculated for 7.83 grams are 3.36 grams and 4.30 grams respectively, making for Fraction 1, 32.29 grams of leucin, 4.30 grams of valin, 0.84 or 2.2 per cent being lost in separation.

<sup>1</sup> *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 2320, 1906.

<sup>2</sup> The following table shows the various rotations obtained by several authors for analytically pure samples of natural valin.

AUTHOR	SOURCE OF VALIN	ROTATION IN 20% HCl	REFERENCE
		<i>degrees.</i>	
E. Fischer .....	Casein.....	+27.95	<i>Zeitschr. f. physiol. Chem.</i> , xxxiii, p. 165, 1901.
Fischer and Dorpinghaus.	Horn.....	+25.9	<i>Zeitschr. f. physiol. Chem.</i> , xxxvi, p. 469, 1902.
Schulze and Winterstein..	{ Lupinus Luteus..	+28.2	{ <i>Zeitschr. f. physiol. Chem.</i> , xxxv, p. 301.
	{ Lupinus Albus..	+27.9	
Abderhalden.....	Edestin.....	+26.7	<i>Zeitschr. f. physiol. Chem.</i> , xl, p. 249, 1903.
T. B. Osborne .....	Phaseolin.....	{ +23.74	{ <i>Zeitschr. f. anal. Chem.</i> , xlviii, p. 102, 1909.
		+24.66	
	Glutinin.....	+25.63	

*Fraction 2.* 8.32 grams, 52.74 per cent C. The substance was dissolved in 50 cc. of water plus 10 cc. of ammonia, and precipitated with 20 cc. of  $\frac{M}{1}$  lead acetate; 6.96 grams of lead salt were obtained, equivalent to 3.91 grams of leucin.

Analysis: 0.4161 gm. substance; 0.2690 gm.  $\text{PbSO}_4$ ; 44.16 per cent Pb.  
Calculated for  $\text{Pb}(\text{C}_6\text{H}_{11}\text{O}_2\text{N})_2$ : 44.29 per cent Pb.

The filtrate yielded 4.32 grams of somewhat impure valin. The substance was recrystallized from dilute alcohol, 3.80 grams being regained of the following composition.

0.1696 gm. substance; 0.2968 gm.  $\text{CO}_2$ ; 0.1416 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C.....	51.24 per cent.	51.65 per cent.
H.....	9.47 "	9.33 "

Rotation of valin in 20 per cent hydrochloric acid: 0.6704 gram substance; 16.757 gram total solution; sp. gr., 1.10; observed in 0.865 dm. tube, + 1.02°.

$$[\alpha]_D^{20} = + 26.8^\circ$$

The mother liquors evaporated to dryness yielded 0.3 gram of mixture of valin with presumably alanin, with 49.83 per cent C, 8.73 per cent H. Fraction 2 consequently yielded 3.91 grams of leucin, 3.80 grams of valin, 0.30 gram of impure valin, 0.2 gram being lost in manipulation.

*Fraction 3.* 8.13 grams, 53.52 per cent C. The substance was dissolved in 50 cc. of water plus 12 cc. of ammonia, and precipitated by 21 cc. of  $\frac{M}{1}$  lead acetate. 9.00 grams of lead-leucin equivalent to 5.05 grams of leucin, were obtained.

Analysis: 0.3170 gm. substance; 0.2045 gm.  $\text{PbSO}_4$ ; 44.06 per cent Pb.  
Calculated for  $\text{Pb}(\text{C}_6\text{H}_{11}\text{O}_2\text{N})_2$ : 44.29 per cent Pb.

From the filtrate 2.92 grams of valin were regained.

Analysis: 0.1201 gram substance; 0.2264 gram  $\text{CO}_2$ ; 0.1031 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C.....	51.24 per cent.	51.41 per cent.
H.....	9.47 "	9.60 "

Rotation in 20 per cent hydrochloric acid; 0.4817 gram substance; 11.225 grams solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.07°.

$$[\alpha]_D^{20} = + 26.2^\circ$$

Fraction 3 yielded 5.05 grams of leucin, 2.92 grams of valin, 0.16 gram being lost in manipulation.

*Fraction 4.* 12.54 grams, 52.79 per cent C. The substance was dissolved in 80 cc. of water plus 20 cc. of ammonia and precipitated with 25 cc. of  $\frac{M}{1}$  lead acetate, equivalent to 6.5 grams of leucin. 8.96 grams of lead-leucin, equivalent to 5.03 grams of leucin, were obtained.

Analysis: (1) 0.3323 gm. substance; 0.2153 gm.  $\text{PbSO}_4$ ; 44.25 per cent Pb.

(2) 0.2274 " " 0.1477 " " 44.36 " "  
Calculated for  $\text{Pb} (\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2$ ; 44.29 per cent Pb.

The filtrate yielded 7.32 grams of valin.

Analysis: 0.1507 gram substance; 0.2844 gram  $\text{CO}_2$ ; 0.1269 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C.....	51.24 per cent	51.45 per cent.
H.....	9.47 "	9.42 "

Rotation of valin in 20 per cent hydrochlorate acid; 0.4972 gram substance; 11.129 grams total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.14°.

$$[\alpha]_D^{20} = + 26.8^\circ$$

Fraction 4 yielded 5.05 grams of leucin and 7.32 grams of valin, a total of 12.37 grams from the original 12.54 grams of mixture.

*Fraction 5.* 7.50 grams. This entire fraction had the composition of valin.

0.1838 gram substance; 0.3440 gram  $\text{CO}_2$ ; 0.1543 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
C.....	51.24 per cent.	51.28 per cent,
H.....	9.47 "	9.39 "

*Fraction 6.* 0.48 gram, 47.13 per cent C., 8.55 per cent H. This fraction was probably a mixture of valin and alanin. It was discarded.

The results of the above separation are collected in the following table:

FRACTION.	WEIGHT.	LEUCIN AND ISOLEUCIN.	VALIN.	TOTAL LEUCIN AND VALIN.	LOSS AND IMPURITIES.
1	37.43	32.29	4.30	36.59	0.84
2	8.32	3.91	3.80	7.71	0.61
3	8.13	5.05	2.92	7.97	0.16
4	12.54	5.03	7.32	12.35	0.19
5	7.50		7.50	7.50	0.00
Total	73.92	46.28	25.84	72.12	1.80

97.6 per cent of the total amino-acids in the five fractions was regained as analytically pure leucin and valin. But for the unnecessary loss in the first, the yield would have been somewhat higher.

#### SUMMARY.

l-Leucin and d-isoleucin may be separated quantitatively from d-valin by precipitation as the normal lead salt,  $\text{Pb}(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2$ . The relative proportions of the leucin isomers may be determined polarimetrically by the rotation of their analytically pure mixture in 20 per cent hydrochloric acid. An exact analysis of the important leucin fraction of proteins is thus rendered possible for the first time.

For purposes of preparation the leucin isomers obtained free from valin may be separated by Ehrlich's method of extracting their copper salts with methyl alcohol. The formerly difficult preparation in quantity of natural d-valin, l-leucin, and d-isoleucin is rendered comparatively simple.

Isoleucin preparations from casein and edestin showed, after repeated purification by means of the lead and copper salts, and by recrystallization, specific rotations of  $+37.35^\circ$  and  $+37.44^\circ$  respectively in 20 per cent hydrochloric acid. The figure  $+37.4^\circ$  doubtless more nearly expresses the rotation of pure natural isoleucin than  $+36.8^\circ$ , the slightly lower figure found by Ehrlich, who had less complete means of purifying the substance.

# ON THE ACTION OF SOAPS UPON THE VITALITY AND IMMUNIZING PROPERTY OF BACILLUS TUBERCULOSIS.

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The bactericidal action of the various body-fluids is one of the incontrovertible facts developed by the modern study of bacteriology. The nature of the bactericidal substances in lymph and blood-serum has been established biologically, but not chemically. We are still ignorant of the precise nature of the bactericidal substances, although we are acquainted with many of their properties. Nor is this to be wondered at, when we consider the great variety of substances contained in the blood-plasma and the great complexity and lability of many of them. The tissue-cells are also bactericidal. That property is possessed by certain motile cells (leukocytes) which form at some stage of their existence parts of certain tissues (bone-marrow) and certain fixed cells (endothelia) which are chiefly fixed in all parts of the body, although from them motile phagocytic elements may possibly be derived.

Lymph and blood-serum are actively bactericidal under conditions which are not strictly living ones, while phagocytes are bactericidal under strictly living conditions. On the other hand, many other body-cells than these capable of acting as phagocytes possess bactericidal properties which come to be exhibited especially after their death. In other words, the dissolution of cells is associated with an increased or renewed bactericidal power. This post-mortem bactericidal power is best exhibited by organs which are permitted to undergo aseptic autolysis. The products formed in this process possess bactericidal properties of high degree.

Conradi, who was the first to note the bactericidal effects of autolyzed tissues, describes the active substances as coctostabile, devoid of reducing power, filtrable through porcelain, unabsorbable by animal charcoal, starch and lycopodium, diffusible through parchment, and soluble in alcohol, from which solutions it is precipitable by ether. In addition, it was said to give the various positive tests for aromatic proteid derivatives. The actual active body could not be secured.

Bartel and his co-workers ascertained that the autolytic products derived from lymphatic tissues were active upon tubercle bacilli, which they attenuated in virulence when permitted to act for many days. Both the human

and bovine types of bacilli were thus acted upon by autolyzed lymphatic tissue; and animals injected with such avirulent or feebly virulent cultures were often protected from subsequent inoculation of virulent bacilli.

My own studies were led up to, not from a consideration of Conradi's and Bartel's work particularly, but from a previous study of the complement-like property of soaps in hemolysis and bacteriolysis, concerning which I have already written. I had found that soaps were extremely injurious to certain bacteria, among which were *B. typhosus* and *B. anthracis*, and that the bactericidal property was completely restrained by inactivated blood-serum. In choosing the tubercle bacillus upon which to test further the soaps, I was influenced by the notion that the fatty or waxy envelop of the tubercle bacillus might be penetrated by the soaps rather better than by other agents in aqueous solution, and the injury might be more subject to graduation and control. This conjecture proved to be well founded, as I will now relate.

When we consider the description of the bactericidal bodies given by Conradi, we may readily identify them as belonging to the soaps in a general way, and the active product employed by Bartel surely contained various soaps. The wide dissemination of fats, lipoids, and lipolytic enzymes in the tissues provides the very conditions for the free production of fatty acids during the catabolic changes in tissues; and the coincident activities, under these conditions, of the proteolytic ferments of the tissues supply the organic bases for union of the liberated fatty acids into soaps, a part of which also unite with inorganic bases in the tissues; and the gradual chemical cleavage, in air and light, of higher fats and lipoids into lower fatty acids, also plays a part.

Whether a similar action to the one produced by autolytic extracts ever takes place in living tissues, I am unable to say. We know now that there is going on generally throughout the body a breaking down and building up of fat into and from the fatty acid radicles. It is not established, however, that the soaps exist generally in the humors in such a state of activity to be an efficient cause of bacteriolysis. The facts may be quite otherwise in local foci in which tissue elements, injured by the tubercle bacillus, are degenerating. The autolytic changes going on in tuberculous tissue may conceivably liberate and produce soaps in such amounts as to be actively destructive to the contained bacilli.

In view of these circumstantial possibilities, and a few established pathological and bacteriological facts bearing upon this line of thought, I have undertaken to determine directly whether some soaps, especially the oleic acid compounds, have a bactericidal effect upon *B. tuberculosis*. I have also extended my observations to the possibility of developing artificial immunity in animals treated with the bacilli, after devitalization of the

organism by means of bactericidal oleic soaps. The results are given in the following protocols:

### EXPERIMENTS—GROUP 1.

ANIMAL EXPERIMENTS CONCERNING THE ANTIBACTERIAL PROPERTIES OF OLEATE SOAPS UPON BOVINE AND HUMAN TYPES OF *B. TUBERCULOSIS*.—The antibacterial properties of oleate soaps (sodium, neurin, and ammonium oleates) were tested by inoculating a large amount, usually 0.5 to 1 c.c., of thick emulsion of *B. tuberculosis*, in soap solution, into guinea-pigs. These emulsions were very thick, and were kept for about twenty-four hours at 37° C. before being used for inoculations. In this series of experiments no cultural tests for the vitality of the tubercle bacilli treated with different strengths of various soaps were made, and nothing definite can be said as to whether these antibacterial properties are due to bactericidal action or to attenuating effect of the soaps.

Four strains of bovine type and one of human type were studied. The results obtained show that the virulent strains of *B. tuberculosis* of either type become so modified by these soaps that they often fail to produce tuberculosis in guinea-pigs. In many cases tuberculosis becomes manifest in a much milder way and progresses very slowly. Death, when it occurs, is invariably much later than in the control animals. In the guinea-pigs which escaped the tuberculous infection from the "soaped" bacilli there is unquestionably an immunity developed.

#### BOVINE TYPE.

$\frac{N}{200}$ . *Sodium Oleate and Bovine X.* (Contact twenty-four hours.) 2:11:'07. One guinea-pig inoculated with the "sodium oleate" culture, then treated for several successive alternate days, showed no tuberculous lesions during the observation period of one hundred and fifty-nine days.

$\frac{N}{200}$ . *Neurin Oleate and Bovine X.* (Contact twenty-four hours.) One guinea-pig similarly experimented on. After seventy-five days the animal, which was apparently well, was chloroformed and examined. No tuberculous lesions found.

$\frac{N}{200}$ . *Ammonium Oleate and Bovine X.* (Contact twenty-four hours.) One guinea-pig similarly experimented on. After seventy days it died, showing typical lesions of tuberculosis.

Controls all died of typical tuberculosis in forty-six and sixty-six days respectively. The culture treated with  $\frac{N}{200}$  soap solutions caused no infection or only mild lesions in guinea-pigs.

*One per cent. Sodium Oleate and B. I. Ravenel.* Type doubtful. (Contact twenty-four hours.) 3:12:'07. One guinea-pig injected. Showed no symptoms of tuberculosis. Found to be refractory to a fresh inoculation with virulent culture (not treated with the soap).

*One per cent. Neurin Oleate and B. I. Ravenel.* (Contact twenty-four

hours.) One guinea-pig injected. Died in one hundred and forty-eight days of general tuberculosis.

*One per cent. Ammonium Oleate and B. I. Ravenel. (Contact twenty-four hours.)* One guinea-pig injected. No tuberculous lesions developed. Later experiment showed immunity against fresh injection.

Controls (three) died of tuberculosis in thirty-eight, thirty-five, and thirty-eight days. Immunity seems to have developed in the vaccinated pigs. The "neurin oleate" pig showed a much milder infection than the controls.

*One per cent. Sodium Oleate and Bovine B. (Contact twenty-four hours.)* 3 : 12 : '07. One guinea-pig inoculated with Bovine B. strain, treated with sodium oleate, showed a peanut-sized swelling of lymphatic gland near the site of inoculation after forty-seven days. The pig was chloroformed and examined for the extent of tuberculous lesions. Several small spots on the spleen, but the liver was quite free. From the spleen inoculations were made on two guinea-pigs (4 : 25 : '07), which died of tuberculosis in eleven days and one hundred and four days respectively.

*One per cent. Neurin Oleate and Bovine B. (Contact twenty-four hours.)* One guinea-pig inoculated with Bovine B., treated with neurin oleate, died in fifteen days of non-tuberculous disease.

*One per cent. Ammonium Oleate and Bovine B. (Contact twenty-four hours.)* One guinea-pig injected. It lived several months with some swollen glands, but without abscesses.

Controls died of tuberculosis in twenty and forty-four days respectively. The guinea-pigs inoculated with the "soap" culture showed much milder infection than the controls.

*One per cent. Neurin Oleate and Bacillus H. Ravenel. (Contact twenty-four hours.)* 4 : 6 : '07. Two guinea-pigs inoculated with H. Ravenel strain, treated with neurin oleate, died of tuberculosis in forty-six and ninety days respectively. Controls (three) died in forty, forty, and thirty days. The lesions of the neurin oleate pigs were decidedly much milder than in the control pigs.

*One per cent. Ammonium Oleate and Bacillus H. Ravenel. (Contact twenty-four hours.)* 4 : 6 : '07. Two guinea-pigs inoculated with B. H. Ravenel, treated with ammonium oleate, did not show any tuberculosis. One survived, and the other died of non-tuberculous disease after one hundred and seventy-three days. Three controls died of tuberculosis in thirty to forty days.

The treatment of H. Ravenel strain with ammonium oleate prevented infection in guinea-pigs (probably the bacilli were killed). In case of neurin oleate tuberculosis of milder grade developed.

*Action of oleate soaps on caseous mass of tuberculous lesion of guinea-pig.* 4 : 26 : '07. The material was obtained from guinea-pigs suffering from Bovine B. X. The caseous mass was suspended in saline solution, whence definite quantities were mixed with 1 per cent. solutions of different

soaps. At the end of eighteen hours, inoculations were made in guinea-pigs. Control guinea-pig died in fifty-two days; "sodium oleate" pig died in seventy-two days; "neurin oleate" pig died in ninety-six days; "ammonium oleate" pig escaped infection.

The material treated with 1 per cent. ammonium oleate for eighteen hours failed to produce tuberculosis in guinea-pig. The materials treated with other soaps caused death much later than the untreated material.

#### HUMAN TYPE.

*Neurin Oleate and H. 38.* (Contact twenty-four hours.) 3 : 12 : '07. One guinea-pig inoculated with H. 38 strain, treated with neurin oleate, died of general tuberculosis in forty-two days.

*Sodium Oleate and H. 38.* One guinea-pig treated with sodium oleate died of typical lesions in seventy-seven days.

*Ammonium Oleate and H. 38.* (Contact twenty-four hours.) One guinea-pig similarly treated (with ammonium oleate) died of typical lesions in eighty days.

Three control guinea-pigs died of tuberculosis in fifty-four, thirty-four, and forty-two days respectively. H. 38 strain seems to be more resistant to the bactericidal properties of oleate soaps.

#### DEVELOPMENT OF IMMUNITY.

*H. Ravenel Strain. (Bovine Type.)* Two guinea-pigs inoculated with the H. Ravenel strain, killed with sodium oleate, did not show any tuberculous symptoms within about three months (4 : 6 : '07 to 7 : 3 : '07). These were inoculated with large amount of H. Ravenel culture on 7 : 3 : '07. Two controls were inoculated also. The controls died of typical tuberculosis in thirty-six and twenty-five days respectively. On the other hand, the vaccinated guinea-pigs remained well for at least fifty days, then emaciation started. Both missing on my return from vacation.

*B. I. Ravenel Strain. (Bovine Type.)* Two guinea-pigs, inoculated with the soaped culture, on 3 : 12 : '07, were still healthy on 7 : 3 : '07. These were inoculated with fresh culture of B. I. Ravenel strain on 7 : 3 : '07. Two control pigs were inoculated at the same time. After a period of over three months one died of non-tuberculous disease. Autopsy did not reveal any foci of tuberculosis. The second pig still remains unaffected by tuberculosis. The two controls died of typical tuberculosis in twenty-two days and in thirty-six days respectively.

These instances are the few in which immunity seems to have developed beyond any question.

#### EXPERIMENTS—GROUP 2.

CULTURAL AND ANIMAL EXPERIMENTS CONCERNING THE ANTIBACTERIAL PROPERTIES OF VARIOUS OLEATE SOAPS AND THEIR COMPONENTS UPON B. TUBERCULOSIS.—Three strains of bovine type, one strain of human type, one strain of frog tuberculosis, one strain of avian type, and one strain of fish tuberculosis were studied.

The actions of soaps were tested either (1) by mixing them with suitable nutrient media and inoculating the virulent, vigorously growing strains on these media, and (2) by pouring solutions of these soaps into the growing cultures of the tubercle bacilli on veal bouillon glycerin-agar slants until the colonies were completely immersed under a deep layer of soap solution. Then, at the end of varying lengths of time, the colonies were fished out, and, after being suspended in saline solution for some hours to liberate the soaps, the bacilli were inoculated upon suitable media (no soaps, of course) and also into guinea-pigs.

The results obtained show that these soaps are actively bactericidal and can devitalize the tubercle bacilli completely if applied in adequate concentrations. Sodium oleate appears to be the most active agent. The constituent components of oleate soaps are unable to kill the bacilli when used in concentrations (calculated to normality) corresponding to the lower effective concentrations of the soaps. Pure oleic acid seems to have a marked bactericidal power if allowed to act for a long time. Sodium hydroxid in  $\frac{N}{40}$  has only slight injurious action upon the bacilli, whereas sodium oleate destroys them in that concentration. In some instances animal experiments were positive, whereas cultural tests failed to get a growth with "soaped bacilli."

#### BACTERICIDAL POWER OF OLEATE SOAP AND ITS COMPONENTS.

*Soaking (immersing) the growing culture in sodium oleate, sodium hydroxid, and oleic acid.* Slant cultures of Bovine B. strain, grown on veal bouillon glycerin-agar for one month (7 : 6 : '07 to 8 : 6 : '07), were filled up with 2 per cent. sodium oleate, or 0.4 per cent. sodium hydroxid, or 2.84 per cent. oleic acid emulsion. At the end of one day, six days, and fourteen days, transplantations were made to regular veal bouillon glycerin-agar slants. The results obtained were as follows:

##### CULTURAL TESTS.

##### *Sodium oleate immersion (2 per cent.):*

1 day's contact	No growth after 40 days.
6 days' "	No growth after 34 days.
14 days' "	No growth after 20 days.

##### *Sodium hydroxid immersion (0.4 per cent.):*

1 day's contact	Grown well after 40 days.
6 days' "	Grown poorly after 34 days.
14 days' "	Grown poorly after 20 days.

##### *Oleic acid emulsion immersion (2.84 per cent.):*

1 day's contact	Grown poorly after 40 days.
6 days' "	Growth doubtful after 34 days.
14 days' "	No growth after 20 days.

## BACTERICIDAL POWER OF OLEATE SOAP.

*(Veal bouillon glycerin-agar slants made July 24, 1907.)*

	BOVINE TYPE.			HUMAN TYPE.
	H. Ravenel.	B. I. Ravenel.	Bov. B.	H. 38.
Two per cent. sodium oleate 1 c.c., to agar 6 c.c.....	8 : 5. No growth.	8 : 5. No growth.	8 : 5. No growth.	8 : 5. No growth.
	8 : 8. No growth.	8 : 8. No growth.	8 : 8. No growth.	8 : 8. No growth.
	9 : 27. No growth.	9 : 29. One tube sterile. One tube few small colonies.	9 : 29. Few small restricted colonies.	9 : 29. A few single colonies.
0.3 c.c.....	8 : 5. Slight growth.	8 : 5. Slight growth.	8 : 5. Slight growth.	8 : 5. Slight growth.
	9 : 29. Much restricted growth. Colonies very thick.	9 : 29. Growth fair, but less than control.	9 : 29. Restricted colonies.	9 : 29. Restricted growth.
0.1 c.c.....	8 : 5. Good growth.	8 : 5. Good growth.	8 : 5. Good growth.	8 : 5. Good growth.
Control.....	8 : 5. Good growth.	8 : 5. Good growth.	8 : 5. Good growth.	8 : 5. Good growth.

Bacillus H. Ravenel did not grow on the first series at all, while the rest grew after three or four weeks in restricted forms. The addition of 0.3 c.c. of 2 per cent. oleate soap solution had a certain inhibitory action, which, after one month, was overcome by the bacilli to a great extent.

## BACTERICIDAL POWER OF OLEATE SOAPS AND OF CALCIUM CHLORID.

*(Veal bouillon glycerin-agar slants made July 1, 1907.)*

	BOVINE TYPE.		HUMAN TYPE.
	H. Ravenel.	B. I. Ravenel.	H. 38.
Five per cent. sodium oleate 1 c.c., to agar 6 c.c.....	7 : 29—28 days. No growth.	No growth.	No growth.
Five per cent. calcium oleate 1 c.c., to agar 6 c.c.....	7 : 29—28 days. No growth.	No growth.	No growth.
Five per cent. calcium chloride 1 c.c., to agar 6 c.c.....	7 : 29—28 days. Good growth.	Good growth.	Good growth.
Control.....	7 : 29—28 days. Good growth.	Good growth.	Good growth.

(Veal bouillon glycerin-agar slants made July 9, 1907.)

	BOVINE TYPES.			HUMAN TYPE.
	H. Ravenel.	B. I. Ravenel.	Bovine B.	H. 38.
Two per cent. sodium oleate 2 c.c.....	7:29—20 days. No growth.	No growth.	No growth.	No growth.
1 c.c.....	No growth.	No growth.	No growth.	No growth.
1.2 per cent. calcium oleate 1 c.c.	7:29—20 days. No growth.	No growth.	No growth.	No growth.
1.2 per cent. calcium chloride 1 c.c.....	7:29—20 days. Good growth.	Good growth.	Good growth.	Good growth.
Control.....	7:29—20 days. Good growth.	Good growth.	Good growth.	Good growth.

## EXPERIMENTS—GROUP 3.

BACTERICIDAL POWER OF OLEATE SOAP.—*Effects of Sodium Oleate Solution upon the Growing Cultures.*—*Bacillus H. Ravenel* (very virulent strain of bovine type) was grown on veal bouillon glycerin-agar slants for three weeks, then the cultures were covered with soap solution of varying concentrations. Transplantations were made from time to time into new soap-free media for the purpose of determining the viability of the soaped *B. tuberculosis*.

Transplanted July 29, 1907, after one to seven days' immersion.

	TWENTY-FOUR HOURS.	SEVEN DAYS.
Two per cent. sodium oleate solution .....	9:29—2 months. No growth.	9:29—2 months. No growth.
0.5 per cent.....	No growth.	No growth.
0.2 per cent.....	No growth.	No growth.
0.04 per cent.....	8:15—17 days. Poor growth.	8:15—17 days. Poor growth.
	9:29—2 months. Good growth.	9:29—2 months. Good growth.
Control in saline solution..	8:15—17 days. Good growth.	8:15—17 days. Good growth.

The virulence of the soaped cultures was tested on guinea-pigs at the end of seven days.

*Animal Experiments with the Soaped Cultures.*—*B. tuberculosis H. Ravenel* was treated with solutions of sodium oleate for seven days and then inoculated subcutaneously near the inguinal region.

*Culture treated with 2 per cent. sodium oleate:*

Three guinea-pigs all survived, showed no swelling of glands, etc. Killed for examination after two months. Showed no tuberculous lesions.

*Sodium oleate treatment (0.5 per cent.):*

Three guinea-pigs all survived until 10 : 24 : '07, when they were found to be free from tuberculous lesions.

*Sodium oleate\* treatment (0.2 per cent.):*

Three pigs all died of tuberculosis in forty, forty-three, and forty-six days respectively. Autopsies revealed typical lesions.

*Sodium oleate treatment (0.04 per cent.):*

Three pigs all died of tuberculosis in twenty-five, twenty-eight, and forty-seven days respectively.

*Saline solution treatment (controls):*

Three pigs all died of tuberculosis in forty-eight, thirty-two, and twenty-eight days respectively.

## EXPERIMENTS—GROUP 4.

*Effects of oleate soaps upon viability and virulence of B. tuberculosis (Bovine B.).*

## (A) CULTURAL TESTS.

Veal bouillon with 0.3 per cent. of oleate soaps becomes so modified that flaky colonies of *B. tuberculosis* from other bouillon culture (without soaps) cannot be floated. In order to prevent sinking of the transplanted colonies in the soap-containing bouillon, I employed disks of cork, upon which the colonies were placed. After seventy-one days (4 : 29 to 7 : 9 : '07) the cultures were examined and found to show no sign of development. In the control there was some growth. From each culture a new culture was made on veal bouillon glycerin-agar slant, with a view to ascertaining their vitality. The following results were obtained:

*Sodium oleate bouillon culture (0.3 per cent.):*

No growth in thirty days after transplantation.

*Ammonium oleate bouillon cultures (0.3 per cent.):*

No growth in thirty days after transplantation.

*Neurin oleate bouillon culture (0.3 per cent.):*

No growth in thirty days after transplantation.

*Oleic acid bouillon culture (0.3 per cent.):*

Moderate growth in fourteen days. Good growth in thirty days.

*Plain (control) bouillon culture:*

Moderate growth in fourteen days. Good growth in thirty days.

Animal experiments were made with these soaped cultures, and they were found to be still alive.

## (B) ANIMAL EXPERIMENTS.

Cultures from sodium oleate bouillon, ammonium oleate bouillon, and neurin oleate bouillon (each, 0.3 per cent.) were able to produce glandular

\* No culture was obtained from this tube.

swellings and death in guinea-pigs. Death occurred within seventy-four, seventy-eight, eighty-two, and one hundred and three days. Guinea-pigs receiving the bacilli from soap-free bouillon culture died within fifty-one and forty-nine days. There were considerable differences between the severity of tuberculous lesions in the two series of animals.

Bovine B. strain is less virulent than H. Ravenel.

### EXPERIMENTS—GROUP 5.

*Soaking the growing culture of B. tuberculosis (H. Ravenel, bovine type) in sodium oleate, sodium hydroxid, and oleic acid.*

Soaking started on August 15th and continued until August 20, 1907. At the end of five days transplantations were made into veal bouillon glycerin-agar slants. The results were as follows:

2 per cent. *sodium oleate* (ca.  $\frac{N}{18}$ )...No growth (9:27) = 37 days.  
0.2 per cent. *sodium oleate* (ca.  $\frac{N}{180}$ )...No growth (9:27) = 37 days.

4 per cent. *sodium hydroxid* ( $\frac{N}{4}$ )...No growth (9:27) = 37 days.  
0.4 per cent. *sodium hydroxid* ( $\frac{N}{10}$ )...Poor growth (9:27) = 37 days.  
0.04 per cent. *sodium hydroxid* ( $\frac{N}{100}$ )...Poor growth (9:27) = 37 days.

*Pure oleic acid* .....Growth doubtful (9:27) = 37 days.

0.9 per cent. *saline solution* .....Good growth (9:27) = 37 days.

This series of experiments shows that oleic acid and sodium hydroxid have much less active bactericidal powers than sodium oleate, which was found to be very active here.

### ANIMAL EXPERIMENTS.

*Tests for vitality of the foregoing cultures after the soaking in soap and other solutions (H. Ravenel strain):*

(1) 2 per cent. *sodium oleate treatment* (soaking) for five days.

August 20th. Five guinea-pigs were inoculated with the cultures (very large quantity) subcutaneously. All but one survived. No symptoms developed up to four months. One pig died in eight days of non-tuberculous cause.

(2) 0.2 per cent. *sodium oleate treatment* (soaking) for five days.

August 20th. Five guinea-pigs were inoculated. Some swollen glands in the inguinal region were observed in all pigs after thirty-six days. Pigs gained weight in the mean time. After two months some abscesses formed, without showing general infection. During the winter many died of non-tuberculous diseases (epidemic). Autopsies showed some small disseminated tuberculous nodules on spleen and liver.

(3) *Pure oleic acid treatment* (soaking) for five days.

August 20th. Three guinea-pigs were inoculated. One died of unknown cause (not tuberculosis); two survived as long as sixty days.

(4) *Sodium hydroxid treatment* (soaking) for five days.

4 per cent. solution. Four guinea-pigs were inoculated. All died of tuberculosis after two to three months. Abscesses of the glands were constant in these cases.

0.4 per cent. solution. Three guinea-pigs were inoculated. All died of tuberculosis. Average in fifty days.

0.04 per cent. solution. Three guinea-pigs inoculated died in thirty-six, forty-five, and twenty-nine days respectively.

(5) *Saline solution* (soaking) for five days.

Four guinea-pigs inoculated. All died in thirty-one, thirty-six, twenty-five, and twenty days respectively.

## EXPERIMENTS—GROUP 6.

To 6 c.c. of veal bouillon glycerin-agar varying quantities of sodium oleate, oleic acid, and sodium hydroxid were added and solidified in slant position. Inoculation was made with H. Ravenel strain (bouillon culture growing for one month).

8:6:'07. Amount per tube, 6 c.c. each .....			
2 c.c. ....	Two per cent. sodium oleate ( $\frac{N}{16}$ ). 8:15—9 days. No growth. 9:27—52 days. No growth.	Sodium hydroxid 0.4 per cent. ( $\frac{N}{16}$ ). 8:15—9 days. Good growth. 9:27—52 days. Lux- uriant growth.	Oleic acid 2.84 per cent. ( $\frac{N}{16}$ ). 8:15—9 days. Growth doubtful. 9:27—52 days. Good growth somewhat restricted.
1 c.c. ....	8:15—9 days. No growth. 9:27—52 days. One tube sterile.	8:15—9 days. Good growth. 9:27—52 days. Like controls.	8:15—9 days. Good growth. 9:27—52 days. Good growth, but less than control.
0.4 c.c. ....	8:15—9 days. Growth doubtful. 9:27—52 days. Sev- eral highly re- stricted colonies grown.	8:15—9 days. Good growth. 9:27—5 days. Like controls.	8:15—9 days. Good growth. 9:27—52 days. Cov- ered with continu- ous colonies.
Control .....	8:15—9 days. Good growth. 9:27—52 days. Cov- ered with continu- ous colonies.	..  ..	..  ..

Sodium oleate in ratio of 2 per cent. solution to culture-media 6 c.c. (equal about  $\frac{N}{60}$ ) stops growth of *B. tuberculosis*, but sodium hydroxid or oleic acid in  $\frac{N}{40}$  cannot stop it.

## ANTIBACTERIAL PROPERTIES OF OLEATE SOAP AND ITS COMPONENTS.

Strains tested were avian, fish, and frog tubercle bacilli, the technic being similar to that in the foregoing series with bovine tubercle bacilli.

Inoculation made August 6, 1907.  
Observation ended October 23, 1907.

	2 PER CENT. SODIUM OLEATE.				0.4 PER CENT. SODIUM HYDROXID.				2.84 PER CENT. OLEIC ACID.			
	2 c.c.	1 c.c.	0.3 c.c.	0	2 c.c.	1 c.c.	0.3 c.c.	0	2 c.c.	1 c.c.	0.3 c.c.	0
Avian tubercle bacilli.....	—	—+	+	+++	+++	+++	+++	+++	+	++	+++	+++
Plagues tubercle bacilli.....	—	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Frog tubercle bacilli.....	—	+	++	+++	+++	+++	+++	+++	++	+++	+++	+++
Bovine B.....	—	—	+	+++	+++	+++	+++	+++	++	+++	+++	+++

Sodium oleate exerts less inhibitory influence upon the growth of *B. tuberculosis* of cold-blooded animals than upon those of warm-blooded animals. NaOH and oleic acid alone are almost inactive when used in corresponding concentrations.

### RÉSUMÉ.

As will be seen from the results presented in the foregoing experiments, the various salts of oleic acid possess a marked bactericidal property on various types of *B. tuberculosis*. It must be noted that the bactericidal property of these salts (or soaps) are not inherent in their component constituents to the same degree as in the compounds. From this it seems probable that the superior antibacterial activity of these soaps is to be ascribed to the changes in physical properties which accompany the formation of oleic soaps, namely, acquisition of higher permeability toward the wax-like coat of the bacillus.

The vaccination of guinea-pigs with the tubercle bacilli devitalized with oleic soaps develops in these animals a complete or partial resistance to a subsequent inoculation with a virulent culture of the same strain of *B. tuberculosis*. In short, a state of immunity against *B. tuberculosis* can be produced in guinea-pigs by means of injections of bacillary emulsion killed by oleic soaps.

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## THE LIFE-SAVING ACTION OF PHYSOSTIGMIN IN POISON- ING BY MAGNESIUM SALTS

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### INTRODUCTION

While studying the inhibitory effect of magnesium salts upon the intestinal contractions produced by intravenous injection of physostigmin, Meltzer and Auer<sup>1</sup> made incidentally the observation that the fibrillary muscular contractions, caused by the physostigmin, were also inhibited. In a subsequent paper by Matthews and Jackson<sup>2</sup> the statement was made that "physostigmin still produced the muscular tremors of the skeletal muscles in general, even after repeated large injections of magnesium sulphate, sufficient to completely stop the heart so that it had to be kept beating by means of electrical stimulation." In order to clear up this contradiction, one of us (Joseph) made the question of the action of magnesium upon physostigmin the subject of a special investigation.<sup>3</sup> These experiments brought overwhelming evidence for the fact that magnesium sulphate is capable of inhibiting the muscular tremor of physostigmin. By large doses of the salt the tremor is abolished completely and permanently; smaller doses may cause only a temporary abolition or reduction of the tremor. It is, however, indispensable for the success of the experiments that the magnesium salt should not be administered in either such strong concentration or with such rapidity that the heart becomes paralyzed before it can effectively distribute the salt solutions to the peripheral organs.

<sup>1</sup> Meltzer and Auer: Amer. Journ. of Physiol., xvii, 320, 1906-1907.

<sup>2</sup> Matthews and Jackson: xix, 12, 1907.

<sup>3</sup> Joseph: Amer. Journ. of Physiol., xxiii, 215, 1909.

Besides the inhibition of the tremor it became evident in the research mentioned that under certain conditions magnesium salts could act as an antidote for the fatal effects of physostigmin, i. e., it was seen that by careful administration of these salts animals survived surely fatal doses of physostigmin.

In that investigation, then, it was established that magnesium salts antagonize some of the effects of physostigmin. The following question presented itself: Is this antagonism mutual, i. e., can some effects of the magnesium salts be antagonized by non-fatal doses of physostigmin? The present communication brings the answer to this question.

*Method.* In this research the experiments were made on rabbits only. In most experiments magnesium sulphate was used, and was administered in molecular concentration by three methods: (a) intramuscularly in the lumbar region, (b) subcutaneously in the back, and (c) into the subscapular space. In the majority of these experiments the first method was used. In a number of other experiments, magnesium chloride in  $\frac{M}{8}$  solution was injected through a cannula into the jugular vein.<sup>4</sup> Physostigmin was administered either through the ear vein or intramuscularly, mostly the latter.

In a number of experiments respiratory tracings were obtained by means of a pneumograph fastened to the epigastrium. In a few instances a graphic record of the respiration was obtained from the pleural cavity by means of the Meltzer cannula. In a few other experiments in which the actions of  $MgCl_2$  and physostigmin upon the motor nerves were studied, one sciatic nerve was exposed and cut, and the peripheral end stimulated with an induction current. The spreading of the toes was taken as an indicator of the motor effect.

#### EXPERIMENTAL RESULTS

In a large majority of the experiments two animals were employed: one animal received magnesium only (control), while another animal received physostigmin shortly after the injection of the magnesium solution. The most demonstrative and therefore the most desirable outcome of such an experiment is a fatal termination in the case of the control animal and a survival of the animal which received physostig-

<sup>4</sup> During all preparatory operations the animals were etherized.

min after the injection of the magnesium solution. The requisite for such an experiment is the employment of a surely fatal dose of the magnesium salt. Here we were confronted with some difficulties at the beginning of the investigation. From the numerous experiments of Meltzer and Auer it seemed evident that a dose of 2 grams of  $\text{MgSO}_4$  per kilo body weight of the animal is certain to be fatal by subcutaneous injection. In our present investigation, however, it was found that the subcutaneous mode of administration was not the most appropriate method for our purpose, as it yielded variable results. In some instances the animals died very soon after an injection of a quantity of the solution equal to 2 grams of the salt per kilo body weight. In other cases quite a long interval passed between the injection and the fatal termination, and in exceptional cases the animal even recovered.

From the experiments with subcutaneous injections of  $\text{MgSO}_4$  we shall mention the following results. Of four rabbits which received physostigmin, three had injections of 2 grams and one of  $2\frac{1}{4}$  grams of the magnesium salt per kilo body weight; *all four animals recovered*. Of three *control* rabbits, each one of which received 2 grams of the salt per kilo body weight, *two died and one survived*. (Two hours and a half after the injection of the salt, while already recovering, the latter received some physostigmin.) The following abbreviated protocols will illustrate these results.

*Experiment 1a.* March 6, 1908. Rabbit A (control), gray, male, 1080 grams.

10:52 a. m. Injected subcutaneously into the back  $\text{MgSO}_4$  2 grams per kilo body weight in molecular solution. Massaged.

11:15 Animal dead.

*Experiment 1b.* Same date. Rabbit B, gray, male, 1000 grams.

10:53. Injected subcutaneously into the back  $\text{MgSO}_4$  2 grams per kilo body weight, in molecular solution. Massaged.

11:15. Respiration very shallow, heart fairly good, lid reflex sluggish. Injected through ear vein 1 milligram of *physostigmin*, followed by 2 cc. saline.

11:16. Respiration improved.

11:17. Some slight convulsive movements (coarse fibrillary contractions); respiration very good; cannot stand or sit, no lid reflex.

11:30. Salivation, a few twitchings; no lid reflex; respiration very good.

11:45. Respiration good; no lid reflex; does not react to pressure of tail.

12:15. Lid reflex returning, no response to pressure of tail, respiration good. Pupils normal, scarcely any twitchings, still salivating; *no urination or defecation*.

2:00. Respiration good, lid reflex fair, responds to pressure of tail, tries to raise head, still lying on side; no twitchings.

2:40. Sits erect in crouching position. Animal recovered.

*Experiment 2a.* September 8, 1908. Rabbit A (control), gray, male, 1460 grams.

10:21 a.m. Injected subcutaneously into back  $\text{MgSO}_4$  2 grams per kilo body weight in molecular solution. Massaged.

10:45. Lying on side, cannot get up, lid reflex fairly good, respiration slow, but fair.

11:08. Lid reflex gone, respiration 48, very shallow, heart rapid.

11:45. Respiration 52, shallow, no lid reflex, no response to pressure of tail, heart 216.

2:00. Struggles to get up, but has no use of hind legs, remains on side, extended. Animal apparently recovering.

2:45. Injected through ear vein  $\frac{1}{2}$  milligram of *physostigmin*. Got up in less than one minute, draws hind legs under body, front legs spread apart, nose resting on table.

3:30. Still in the above position. Injected again  $\frac{1}{2}$  milligram of *physostigmin* into ear vein. Immediately raises head, draws in fore legs; much improved. Animal recovered.

*Experiment 2b.* Same date. Rabbit B, gray, male, 1410 grams.

10:20. Injected-subcutaneously into the back  $\text{MgSO}_4$  2 grams per kilo body weight in molecular solution. Massaged.

10:39. Respiration very slow and shallow, lid reflex gone. Injected through ear vein 1 milligram of *physostigmin* followed by 2 cc. saline.

10:40. Respiration improved, tried to get up; fairly strong convulsive movements, no fibrillary contractions.

10:45. Convulsions all stopped, respiration very good, pupils wide.

11:10. Respiration very good, 54 per minute, faint response to pressure of tail; lid reflex returning.

12:20. Sits up in crouching position, holds up head with difficulty, sensation perfect, respiration good.

While even the experiments with subcutaneous injections were thus showing unmistakably the favorable action of *physostigmin* upon the

toxic action of magnesium salts, we were bent upon finding methods of injection capable of yielding more definite results. We attempted at first to inject the magnesium solution into the subscapular space. This method proved indeed to be more advantageous than the simple subcutaneous method of injection. Much smaller doses were needed and the effects were fairly constant. However, we soon abandoned even this method in favor of the intramuscular injection. It was established that a dose of 1.2 gram of  $\text{MgSO}_4$  per kilo body weight given intramuscularly invariably kills a rabbit in a short time.

The experiments carried out by this method gave a definite result indeed. Of seven controls which received by intramuscular injection 1.2 gram per kilo body weight, *all died* and in less than 20 minutes after the injection. The longest interval between injection and death was 18 and the shortest 3 minutes. Of 10 rabbits which received physostigmin soon after the injection of  $\text{MgSO}_4$  (the same dose and in the same manner as in the controls) *nine survived and one died*. Of the nine surviving animals the majority received the physostigmin intramuscularly (in another place than the one in which the magnesium solution was given). The dose was in most cases not more than 1 milligram and in two cases but 0.5 milligram. The injection of physostigmin was given in most cases shortly after the administration of the magnesium salt, especially when the physostigmin also was given intramuscularly.

The following few abbreviated protocols of experiments may serve as illustrations:

*Experiment 3a.* June 26th, 1908. Rabbit A (control), white, female, 2300 grams.

10:21 a.m. Injected intramuscularly 11.2 cc. of a molecular solution of  $\text{MgSO}_4$  (= 1.2 grams per kilo body weight), in the right lumbar region (about  $\frac{1}{2}$  cc. lost).

10:27. No respiration, heart very slow, no lid reflex.

10:29. Dead.

*Experiment 3b.* Same date. Rabbit B, white, female, 1900 grams.

10:22. Injected intramuscularly 9.3 cc.  $\frac{2}{3}$   $\text{MgSO}_4$  (= 1.2 grams per kilo body weight), into right lumbar region.

10:26. Lying on side, respiration very shallow and slow, getting worse very rapidly. Injected 1 milligram of physostigmin intramuscularly into left back.

10:29. Respiration 100, good, regular.

10:35. Pupils small, respiration very shallow and slow blood (ear vessels) very dark, heart slow. Injected  $\frac{1}{2}$  milligram of *physostigmin* into ear vein.

10:41. Respiration very poor, heart slow. Injected again  $\frac{1}{2}$  milligram into ear vein.

10:43. Respiration 60, but still shallow, heart 144, blood good color now.

10:51. Respiration 66, heart 204, regular; strong salivation, no defecation.

11:28. Respiration 82, good, regular, heart 240; animal seems to be in good condition.

12:05. Got up, holds head up part of the time. Animal recovered.

*Experiment 4a.* June 25, 1908. Rabbit A (control), white, 1700 grams.

10:47. Injected intramuscularly  $\text{MgSO}_4$  1.2 grams per kilo body weight.

10:55. Lying on side, respiration good.

11:00. Respiration 52, very shallow:

11:02. Asphyctic movements, no regular respiration, pupils wide.

11:04. Dead.

*Experiment 4b.* Same date. Rabbit B, black, 1700 grams.

10:45. Injected intramuscularly  $\text{MgSO}_4$  1.2 grams per kilo body weight.

10:53. Lying on side, respiration slow. Injected one milligram *physostigmin* in the left lumbar muscles.

10:56. Respiration 116, very good, lid reflex sluggish.

10:58. Same, moderate twitchings.

11:00. No lid reflex, respiration 144.

11:09. Respiration 120, but not very deep.

11:10. Respiration much deeper.

11:17. Respiration very good, 88.

11:55. Slight lid reflex, responds slightly to pressure of tail.

12:45. Got up. Animal recovered.

*Experiment 5a.* June 29, 1908. Rabbit A (control), gray, 1200 grams.

10:20. Injected intramuscularly  $\text{MgSO}_4$  1.2 grams per kilo body weight.

10:22. Lying on side.

10:23. Dead.

*Experiment 5b.* Same date. Rabbit B, gray, 1400 grams.

10:19. Injected intramuscularly  $\text{MgSO}_4$  1.2 grams per kilo.

10:21. Respiration becoming rapid. Injected intramuscularly 1 milligram *physostigmin*.

10:26. On side, respiration rather poor, some convulsive movements

10:40. Respiration regular, efficient, slight lid reflex, lying on side, no voluntary movements.

11:10. Turned over, but cannot hold up head.

11:20. Can hold up head without difficulty. Animal recovered.

*Experiment 5c.* Same date. Rabbit C, gray, 1390 grams.

10:40. Injected intramuscularly  $\text{MgSO}_4$  1.2 grams per kilo.

10:41. Injected intramuscularly  $\frac{1}{2}$  milligram *physostigmin*.

11:00. Still sitting up, but rather shaky. No twitchings.

11:07. Lying on side, respiration 54, good lid reflex.

11:30. Respiration 64, very good, no lid reflex, no response to pressure of tail.

12:07. Respiration good, tries to get up when tail is pinched. Animal recovered.

Even for intramuscular injections it cannot be assumed that the same dose of the magnesium salt will always act exactly in the same manner. It will prove to be more toxic and act more rapidly in one case than in another. Nevertheless a dose of 1.2 gram (per kilo body weight) of  $\text{MgSO}_4$  proved to be invariably fatal, while the additional injection of *physostigmin* saved all animals except one. It seemed that the sooner after the injection of the magnesium salt the *physostigmin* was administered, the safer was the effect, so that with a one-minute interval between the injection of magnesium and *physostigmin* even one-half of a milligram of the latter was sufficient to save the life of the animal.

However, *physostigmin* proved to be life-saving only when the magnesium salt was administered in a minimum lethal dose. When the dose was increased perceptibly above this minimum, the *physostigmin* failed to act as a reliable antidote. Of four rabbits which received intramuscularly 1.3 gram of  $\text{MgSO}_4$  per kilo body weight, only one recovered. But even in the animals with a fatal outcome the retarding effect of *physostigmin* was manifest. While in the control animals, which received only 1.2 gram of the magnesium salt per kilo, the longest time an animal lived after its injection was 18 minutes, the shortest time which the *physostigmin* animals lived after receiving 1.3 gram

per kilo of  $\text{MgSO}_4$  was 37 minutes. The following experiment will demonstrate the favorable action of physostigmin even in unavoidably fatal doses of  $\text{MgSO}_4$ .

*Experiment 6.* Gray rabbit, 1740 grams.

2:58. Injected intramuscularly 1.3 gram of  $\text{MgSO}_4$  per kilo body weight.

2:59. Injected intramuscularly 1 milligram of *physostigmin*.

3:09. Shows effects of *physostigmin*; getting weak, lid reflex active.

3:17. Respiration poor, animal lying on side, no lid reflex.

3:19. Respiration very poor, irregular, heart slow. Injected intramuscularly  $\frac{1}{2}$  milligram of *physostigmin*. Massaged.

3:21. Respiration 36, only moderately strong.

3:27. Respiration 56, efficient, no lid reflex.

3:40. Respiration 48.

4:14. Dead.

This animal lived 76 minutes after the injection of 1.3 gram of the magnesium salt per kilo body weight. One needs only to compare the course of life of this animal subsequent to the injection of  $\text{MgSO}_4$  with the course of life of the above quoted controls which received 1.2 gram per kilo body weight, to recognize clearly the antagonistic action of the *physostigmin* even in the fatal cases.

As stated above, a number of experiments were made by subscapular injections, which gave more uniform results than the experiments with subcutaneous injections. There was some variability in the action of the subscapular injection in that the development of the effect varied with the individual animals, in some appearing earlier and in others later. There was, however, a definite stability regarding the final effect of each dose of the salt. For instance a dose of 1.3 gram of magnesium sulphate was the minimum fatal dose by subscapular injection, but there were individual variations in the rapidity with which the effect took place. The following is an illustrative experiment in which the  $\text{MgSO}_4$  was given subscapularly:

*Experiment 7a.* June 12, 1908. Rabbit A (control), white, 1446 grams.

10:32. Injected subscapularly 1.3 gram  $\text{MgSO}_4$  per kilo body weight.

10:38. Lying on side, respiration fair, good lid reflex.

10:46. Scarcely any respiration, heart very slow.

10:48. No perceptible respiration, pupils wide, heart weak and slow.

10:49. Dead.

*Experiment 7b.* Same date. Rabbit B, gray, female, 1420 grams.

10:30. Injected subscapularly 1.3 gram  $\text{MgSO}_4$  per kilo body weight.

10:35. Fell over on side, going under rapidly.

10:40. Respiration very shallow, lid reflex gone. Injected through ear vein 1 milligram *physostigmin*.

10:41. Some convulsive movements and fibrillary contractions.

10:45. Respiration good, but difficult to judge on account of the convulsive movements (which, however, became less). No lid reflex

10:49. Respiration getting much better, 72, regular and fairly strong. Considerable salivation, fibrillary contractions only slight.

11:00. Respiration 70, strong and regular; no lid reflex and no response to pressure of tail.

12:10. Respiration very good, 54, slight lid reflex, slight response to pressure of tail. Injected intramuscularly 1 milligram of *physostigmin*.

12:22. Responds better to pressure of tail, respiration very good. Animal recovered.

It was thus firmly established that physostigmin was capable of serving as an antidote to a surely fatal amount of  $\text{MgSO}_4$ , if the dose of the latter was not too large. The antagonizing action of the physostigmin was also evident, however, when it was given to animals which were under the manifest influence of a non-fatal dose of magnesium salt. The most striking reaction was the improvement of the respiration which followed upon the administration of the physostigmin. This reaction was plainly recognizable in all experiments whether the physostigmin led to a recovery of the animal from the fatal dose of magnesium or not, or whether the dose of magnesium was not fatal, but only more or less toxic. The favorable effect of physostigmin upon the respiratory depression brought on by magnesium was variously studied by us by graphic methods. The favorable effect was never missing. The following tracings illustrate such effects.

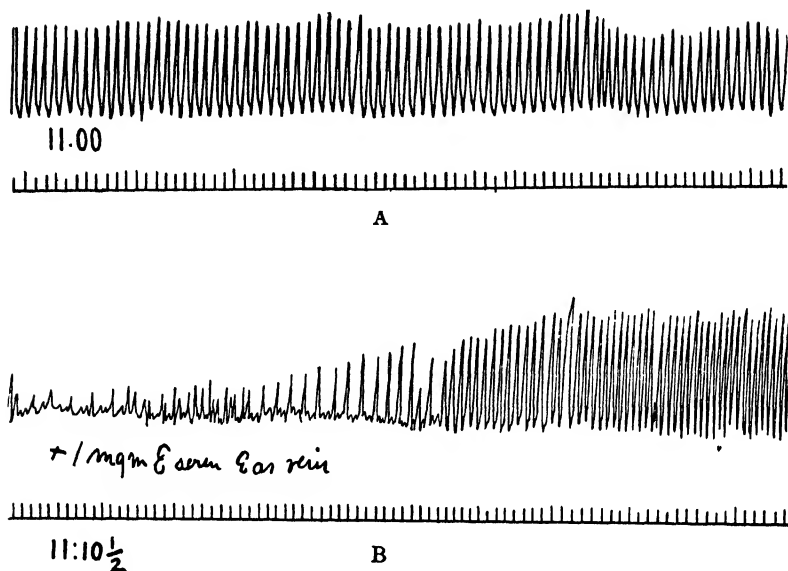


FIG. 1. RESPIRATORY TRACING BY PNEUMOGRAPH, INSPIRATION UPWARDS; READ FROM LEFT TO RIGHT. RABBIT RECEIVED BY SUBCUTANEOUS INJECTION TWO GRAMS  $MgSO_4$  PER KILO. TRACING (A) TAKEN FIVE MINUTES AFTER INJECTION OF MAGNESIUM. TRACING (B) TAKEN FIFTEEN MINUTES AFTER INJECTION OF MAGNESIUM. PHYSOSTIGMIN (ESERIN) INJECTED AT X.

The tracings in both figures were obtained from rabbits which received each 2 grams of  $MgSO_4$  per kilo body weight by subcutaneous injection. Tracing A in fig. I was taken 5 minutes after injection of the magnesium salt. Tracing B of that figure was taken about 10 minutes later. The respiration was very shallow. Immediately after the intravenous injection of 1 milligram of physostigmin the respiration improved and shortly after became even more frequent and deeper than it was at the beginning of the experiment.

Tracing A of fig. II was obtained about 40 minutes after the injection of the magnesium solution. Except for some weak heart beats the animal appeared to be dead. No sign of a respiration was present and artificial respiration was instituted. Then the artificial respiration was stopped and intravenous injection of 1 milligram of physostigmin was given. Immediately the spontaneous respiration began

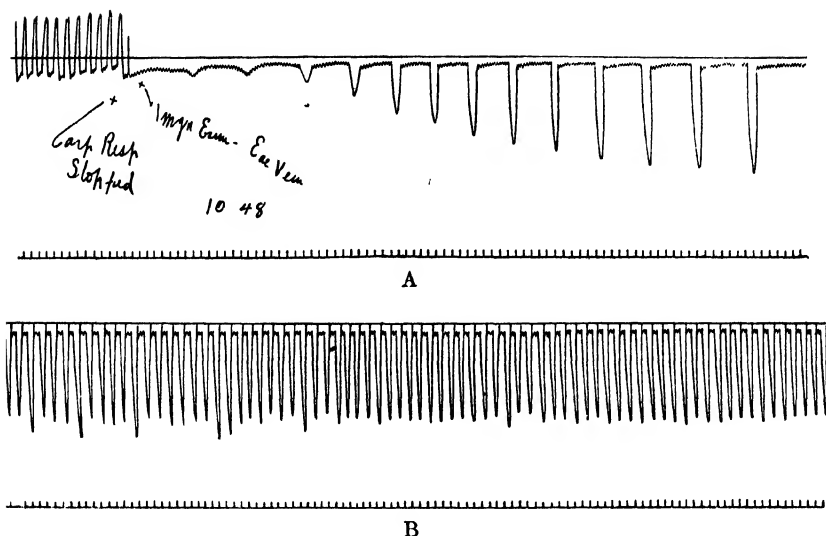


FIG. II. RESPIRATORY TRACING BY PLEURAL CANNULA; INSPIRATION DOWNWARD; READ FROM LEFT TO RIGHT. RABBIT RECEIVED TWO GRAMS OF MAGNESIUM SULPHATE PER KILO. FORTY MINUTES LATER THERE WAS NO RESPIRATION AND ARTIFICIAL RESPIRATION WAS STARTED. TRACING (A) SHOWS FIRST, ARTIFICIAL RESPIRATION, THEN A. R. STOPPED AND PHYSOSTIGMIN INJECTED AT (X). TRACING (B) TAKEN TWENTY MINUTES LATER, SHOWS STRONG SPONTANEOUS RESPIRATION. LINE ABOVE RESPIRATION IN BOTH TRACINGS INDICATES ATMOSPHERIC PRESSURE.

to show up and within a fraction of a minute it obtained a good depth, although it remained for a while fairly slow. Gradually, however, it became also fairly rapid. Tracing B in fig. II is taken 20 minutes later from the same animal. The respirations appeared as frequent and at least as rapid as in a normal rabbit.

The reaction upon the respiration appeared sooner, the respirations became more rapid and deeper and the favorable effect lasted longer, the earlier the physostigmin was administered after the injection of the magnesium salt. When the toxic effect of the magnesium was permitted to take a deep hold before the physostigmin was administered, the favorable effect of the latter was slow in its manifestation, appeared to be less profound and passed off too soon to be of permanent value. Too frequent and too many or too

large doses of physostigmin not only did not offer an additional aid, but on the contrary seemed to add the toxicity of the physostigmin to that of the magnesium salt. However, this last statement is based only on incidental observations—we made no systematic study on this special point.

Besides the manifest influence upon the respiration the injection of physostigmin seems to produce a stimulating reaction on the other paralytic phenomena brought on by magnesium. This effect, as judged by the general symptoms, was rare, insignificant, ephemeral and appeared then in those cases where the dose of magnesium was moderate and produced only light toxic symptoms. Now and then after an injection of physostigmin the animal would seem to be more capable to move one of the extremities, to raise the head or the lid reflex would improve slightly. These improvements, however, would pass off soon even in animals which later recovered with ease. On the contrary, we have seen in this series of experiments as well as in many incidental observations on rabbits and dogs that an injection of physostigmin which improved the respiration and thus assured greatly the life of the animal left it nevertheless in an almost completely relaxed state, without lid reflex and without recognizable sensation; the animal remaining in this state perhaps until it finally began to recover. In fact, *in using a magnesium salt as an anesthetic, it seemed in some cases desirable to add an injection of physostigmin*, as it did not interfere with the general state of the animal while it aided in the protection of its life by supporting the respiration. The antagonistic action of calcium against magnesium differs from that of physostigmin in that the former restores motility and sensation completely, or at least to a great degree, while the latter improves essentially only the respiration leaving the other effects of the magnesium practically untouched.

These conclusions were drawn from the general behavior of the animal under the conditions of the experiments. However, in a direct study of the behavior of the motor nerves under the influence of magnesium salts and physostigmin, it became evident that the restoring action of physostigmin upon the motor function is after all not such a negligible quantity. As stated above, in a few experiments the sciatic nerve was cut and the peripheral end stimulated by a faradic current.

In these animals magnesium chloride was used in an  $\frac{M}{8}$  solution, which ran slowly from a burette into the jugular vein. Before starting the infusion the strength of the electrical stimulus was established, which by application to the peripheral end of the sciatic caused spreading of the toes. After the infusion began, and while it continued, the irritability of the sciatic nerve was again tested at various times before and after an injection of physostigmin. The effect of the physostigmin upon the motor nerve was frequently compared with that upon the respiration. The following greatly abbreviated protocols of these experiments will throw some light upon the points under discussion.

*Experiment 8.* June 24, 1909. Gray male rabbit, 1580 grams. Ether, tracheotomy, cannula in jugular vein connected with burette, exposed and cut both sciatics. Petzold coil armored with one Daniell.

11:30. Toes spread well with 350 mm. coil distance.

11:35. Started infusion of  $\frac{M}{8}$   $MgCl_2$ .

11:43. 14 cc. in.; 350 - 150 c.d. (coil distance) = 0, 100 = slight spreading of toes.

11:50. 26 cc. in.; 150 - 60 c.d. = 0, 40 c.d. = slight. Injected intravenously one mgr. physostigmin.

11:52. 100 c.d. = good spreading of toes. *Distinct improvement.* Respiration good.

12:00. 37 cc in.; 60 c.d. = slight. No respiration, started artificial respiration. Injected again 1 milligram physostigmin.

12:01. 60 c.d. = slight (perhaps a trifle improved). Artificial respiration discontinued: immediately good voluntary respiration.

*Good improvement of respiration; practically no improvement of motor nerve.*

*Experiment 9.* June 29, 1909. Gray male rabbit, 1775 grams. (Preparation as in last experiment).

11:30. 350 c.d. = strong spreading of toes.

11:33. Started infusion of  $\frac{M}{8}$   $MgCl_2$ .

11:43. 18 cc. in.; 100 c.d. = 0; 60 c.d. = faint movement of toes; no voluntary respiration. Artificial respiration.

11:45. Injected 1 milligram physostigmin.

11:46. Voluntary respiration returned. 60 c.d. = faint movement; no improvement.

11:47. 120 c.d. = faint movements of toes (very slight improvement).

From the experiments with stimulations of the motor nerves we learn that physostigmin may indeed restore to a moderate degree the indirect irritability which has been greatly reduced through the action of the magnesium salt. Exceptionally the restoration may be fairly considerable; this seemed to occur mostly before too much of the magnesium was infused. In the advanced stage of the experiment after a fairly good amount of the magnesium had run in, the favorable effect of physostigmin upon the restoration of irritability seemed to be very slight, while at the same time the action upon the respiration might still be very good. When the infusion of  $MgCl_2$  is discontinued, the irritability of the motor nerves returns gradually. During this stage the action of physostigmin may again become more manifest by perceptibly hastening the restoration of the irritability.

At any rate, even in the experiments with direct stimulation of the motor nerves, it was evident that the antagonism of physostigmin to the vital depression of magnesium salts was infinitely greater in the specific action upon the respiration than in the effect upon motor paralysis in general.

The following side issues, observed in this investigation only incidentally, may be mentioned here very briefly. They relate to the behavior of the various toxic effects of physostigmin when administered after the injection of magnesium salts. We should mention in the first place that in not a single case was there any purgative effect which is otherwise so characteristic for the administration of physostigmin. The coarse and fine fibrillary tremors peculiar to the action of this drug were also greatly reduced, although not completely suppressed. These tremors were few, weak and only of short duration, when the physostigmin was administered intramuscularly. When injected intravenously the tremors, especially the coarse ones, were somewhat more pronounced; but even then they could not compare with the tremors which follow an intravenous injection of physostigmin into a normal rabbit. This subject, however, was specially dealt with in the previous paper by Joseph.<sup>5</sup>

After the injections of physostigmin the pupils were never seen to

<sup>5</sup> Joseph: l. c.

have become so small as is sometimes observed after an injection of that drug alone, although the pupil was often quite small before the physostigmin was given, apparently as an effect of the magnesium salt. In fact, in some of the protocols it is noted that the pupils became wider after the injection of the physostigmin. We shall not attempt to discuss at this place the factors which may possibly be concerned in this occurrence. Salivation was present in most of the experiments. We are, of course, unable to state whether that action occurred in its normal extent or was reduced. At any rate salivation seems to have been less affected than any of the other toxic actions of physostigmin.

#### DISCUSSION

The experiments have safely established that physostigmin is capable of antagonizing some of the toxic actions of magnesium salts. It may directly serve as a life-saving agent against a fatal dose of magnesium salt if the dose of the latter is not too large. It improves definitely the respiration in every instance whether the dose of magnesium salt be only toxic or fatal, revocably or irrevocably. Physostigmin apparently exerts some antagonistic influence also upon the general motor paralysis. This influence, however, is very small, especially when compared with that which is exerted upon the respiration. The motor influence, when not tested by special means, is very little in evidence on the animal. While after an injection of physostigmin the respiration becomes strikingly improved and life seems to be assured, the animal may remain for one hour or two completely relaxed without voluntary or reflex movements, without sensation and without lid reflex.

The life-saving influence of physostigmin upon the severely toxic effects of magnesium salt becomes effective apparently through the favorable action of the former upon the respiratory function. How are we to explain this action? It is generally agreed upon, that physostigmin is fatal to the animal by paralyzing the respiratory center. It was established by Meltzer and Auer<sup>a</sup> that the fatal issue in magnesium poisoning is primarily due also to paralysis of the respiratory cen-

<sup>a</sup> Meltzer and Auer: Amer. Journ. of Physiol., xiv, 366, 1905; xv, 387, 1905-1906.

ter. How does it happen that one of two agents which, when acting separately, paralyzes the respiration, should improve this function when administered after the other? The plausible explanation of this apparent contradiction is to be found, so we believe, in the fact that *physostigmin stimulates the respiratory function before it paralyzes it*. It was observed by many students of the action of physostigmin that at an early stage of the poisoning or by a smaller dose the respiration is accelerated. Bezold and Goetz<sup>7</sup> who found that the accelerating action is eliminated if both vagus nerves are cut, assumed that the acceleration of the respiration has its origin in a stimulation of the nerve endings of the vagus within the lungs. Rothberger,<sup>8</sup> who confirmed the observation that the acceleration is greater when the vagi are intact, established further however the fact that even after section of the vagi administration of physostigmin accelerates the respiration. On the basis of his results Rothberger concludes that physostigmin stimulates the peripheral nerve endings of the vagus as well as the respiratory center directly within the medulla. In our case we may assume that the injection of physostigmin overcomes the respiratory depression by its double action: by directly stimulating the center and also by stimulating the respiratory fibers of the vagus within the lungs. In so far as it stimulates the center physostigmin is a real antagonist to magnesium since the latter causes in all doses only an inhibition—a depression of the respiratory center.

However, the antagonistic relations of physostigmin to magnesium have to be discussed from yet another point of view. Magnesium exerts also a depressing effect upon muscle and motor nerve endings or, as it is frequently expressed, upon direct and indirect muscle irritability. The depressing action of magnesium chloride upon the indirect irritability we have recently established in (unpublished) studies upon frog muscles; we shall not enter here into a further discussion of that fact. The depressing action upon the motor nerve ending magnesium shares with most of the other inorganic ions of the animal body. It is the curare-like action which is exerted by sodium chloride,

<sup>7</sup> V. Bezold and Goetz: quoted after Rothberger, *Pflüger's Archiv*, lxxxvii, 117, 1901.

<sup>8</sup> Rothberger: *Pflüger's Archiv*, lxxxvii, 117, 1901.

potassium chloride and, as we have recently shown,<sup>9</sup> also by calcium chloride as a primary effect, although in a secondary action calcium may antagonize this very action of the other ions. Wiki<sup>10</sup> attempted to explain all the toxic effects of magnesium by this curare-like action. We shall not enter here into a discussion of that point. This will be done later at another opportunity. We shall only say briefly that there are many facts which demonstrate the incorrectness of that view. The very fact that under certain circumstances the animal may have a very effective respiration while remaining for hours without voluntary movements and without sensations shows manifestly the sharp difference between the effects of curare and that of magnesium. On the other hand it was found in experiments that the respiration was already completely abolished, while the peripheral ends of the phrenics and other motor nerves still gave a satisfactory response.

However this may be, the "curare-like" action of magnesium upon the peripheral motor nerve endings has surely some share in the danger which comes from the respiratory paralysis. With an increasing depression of the irritability of the respiratory center a weakening of the irritability of the nerve endings of the respiratory muscles, be it ever so small, is a grave factor. Now, Pal<sup>11</sup> discovered that physostigmin is antagonistic to curare. Curare acts, as is known, on the nerve endings. The place of action of physostigmin in the production of the tremor has been a matter of dispute. Harnack and Witkowski<sup>12</sup> believe that it is the muscle tissue itself which is stimulated; Kobert and Schweder<sup>13</sup> believe that it is the nerve endings which are stimulated by physostigmin. The experiments of Rothberger<sup>14</sup> led him, however, to the conclusion that the physostigmin stimulates nerve endings as well as muscle tissue. We have seen in our experiments that physostigmin antagonizes the effects of magnesium also in the action of the latter upon the muscle apparatus. And since magnesium de-

<sup>9</sup> Joseph and Meltzer: *Proceed. of the Soc. f. Experim. Biol. and Med.*, vol. vi, 105, 1907.

<sup>10</sup> M. B. Wiki: *Comt. rend.*, T. cx, 1906

<sup>11</sup> Pal: *Centralbl. f. Physiol.*, xiv, 255, 1900.

<sup>12</sup> Harnack and Witkowski: *Arch. f. experim. Pathol.*, v, 401, 1876

<sup>13</sup> Schweder: after Rothberger, l. c.

<sup>14</sup> Rothberger: l. c.

presses nerve endings and muscle tissue and physostigmin stimulates both, the antagonism in our case might extend to both and might therefore be more extensive than the antagonism between physostigmin and curare, since the action of the latter is confined, as is generally assumed, to the nerve endings alone. However, the intensity of the action of physostigmin against curare is undoubtedly much greater than its action against magnesium, as far as the motor effects of the latter are concerned. While an injection of physostigmin into a curarized animal brings the latter within a short time to its legs, the injection of physostigmin into an animal paralyzed from a magnesium salt has an insignificant effect as far as the motility of the animal is concerned. On the other hand, this antagonism of physostigmin to the curare-like action of magnesium while it is without importance to the general motility is probably of a telling value to the life of the animal since it aids by overcoming the weakness of the respiratory muscles.

Our theory regarding the nature of the life-saving action of physostigmin upon the fatal poisoning by magnesium is therefore that physostigmin aids the endangered function of respiration in three ways; it stimulates the respiratory center; it stimulates muscle and nerve endings of the respiratory muscles—both these actions of physostigmin are truly antagonistic to the actions of magnesium; and lastly physostigmin aids respiration by stimulating the nerve endings of the vagi within the lungs. By this threefold aid the function of respiration succeeds in regaining its own against the continuous depressing action of magnesium. The other mechanisms of the body, however, with only the slight aid which they receive from physostigmin in its antagonism to the action of the magnesium ion, remain practically powerless against the extensive central and peripheral depressing effects of the magnesium salts.

#### SUMMARY

Physostigmin is capable of efficiently antagonizing some of the toxic actions of magnesium salts. It may directly serve as a life-saving agent against a fatal poisoning by magnesium salts, if the dose of the latter employed be not too large.

Physostigmin overcomes the toxic effects of magnesium essentially by the aid it renders to the depressed function of respiration. This aid is of threefold origin. It stimulates the respiratory center; it antagonizes the "curare-like" action of the magnesium-ion upon the nerve endings of the respiratory muscles; and it stimulates the nerve endings of the pneumogastric nerves within the lungs.

Physostigmin antagonizes also the magnesium action upon the peripheral nerve endings and probably also the action upon muscle tissue. The extent of this antagonism, however, seems to be not very significant.



## THE INFLUENCE OF CALCIUM UPON THE PUPIL AND THE PUPILLOMOTOR FIBRES OF THE SYMPATHETIC NERVE.

By JOHN AUER AND S. J. MELTZER.

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for Medical Research.]*

WHILE studying the influence of the salts of calcium and magnesium upon the development of rigor mortis we observed that the pupils of animals which had received intravenously solutions of calcium salts became contracted. Being interested in the effects of calcium upon the various organs and tissues of the animal body, we decided to make this incidentally observed phenomenon the subject of a special investigation. We studied at the same time the influence of calcium upon the cervical sympathetic nerve with reference to the effect of its stimulation upon the size of the pupils. We intend to give in this paper an account of the results we have obtained in these investigations.<sup>1</sup>

**Method.** — While in the studies upon rigor mortis various salts of calcium were employed and the injections were made into various species of animals, in the present series the observations were made only on rabbits, and as a calcium salt the chloride only was employed. The solutions were administered by intravenous injection mostly through the external jugular vein. In a few cases the injections were given through the ear vein. The calcium chloride was invariably employed in  $\frac{1}{8}$  molecular solution. As a rule the animal was etherized, tied on a holder, one external jugular vein exposed and a cannula tied into it, one sympathetic nerve or both were exposed, and then we waited until the animal had sufficiently recovered from the ether. The solution was permitted to run into the vein from a burette at the average rate of about 2 c.c.

<sup>1</sup> Short accounts appeared in the *Proceedings of the Society for Experimental Biology and Medicine*, 1907-1908, v, p. 86, and *Zentralblatt für Physiologie*, 1908, xxii, p. 245.

per minute. When administered through the ear vein, no etherization was necessary, and the injection was accomplished more rapidly. The stimulation of the sympathetic nerve was carried on by means of an induction coil.

The calcium myosis was also studied while the animal was under the influence of such mydriatics as atropin, cocain, and adrenalin; these were administered either by instillation or by intravenous injection either before or after the infusion of calcium chloride. Observations were also made upon the effect of ether and asphyxia on the behavior of the myosis in question.

### THE EXPERIMENTAL RESULTS.

**Calcium myosis.**—An intravenous infusion of calcium chloride caused invariably a narrowing of the pupils. The first unmistakable effects could be already noticed after an infusion of only 10 c.c. of the  $m/8$  solution, and as a rule 22 c.c. or a trifle more would be sufficient to contract the pupil to nearly a pin point. Outside of this striking effect the animal would be awake and might not yet show any definite toxic symptoms, and when the infusion was stopped at this point and the animal was taken off the board and left to itself without further experimentation, it would completely recover. The amount of the calcium solution which sufficed to bring on a maximum myosis varied in general with the size of the animal and the rate of injection, slower injections requiring larger quantities for the same effect. There were, however, very few normal animals in which 30 c.c. of the solution would not bring on a strong constriction of the pupil. With the development of the constriction the pupils gradually lose their susceptibility to light, and neither excitement nor struggles of the animal bring about any change in the width of the pupils. The strongly constricted pupil appears to be absolutely immovable. However, after finishing the infusion and suturing the wound, the removal of the rabbit from the holder seemed to cause in some animals a slight widening of the contracted pupil. This widening as indicated was very slight and mostly only temporary. Furthermore, in a few instances there appeared rather a slight increase of the constriction of the pupil immediately after the removal of the animal from the holder. At any rate, these changes were very slight and ephemeral. As a rule,

after the discontinuation of the infusion of the calcium, whether the animal remained on the holder or was removed from it, the attained constriction of the pupil remained practically unchanged for a period varying between fifteen minutes and an hour and a half or even longer, and further, as a rule, several hours passed before the pupil attained its normal size and its normal reaction to light. The following few abbreviated protocols will illustrate the statements:

*Experiment 1.* — White male rabbit, 1570 gm. Etherized and cannula inserted into left jugular vein.

11.35 A. M. Operation finished and ether discontinued.

11.50 A. M. Both pupils moderately contracted; started infusion of  $\text{CaCl}_2$   $m/8$  from a burette.

11.57 A. M. 14 c. c. in; both pupils very small.

12.00 M. 21 c. c. in; stopped infusion; both pupils very small, nearly pin point.

12.10 P. M. Wound sutured and animal removed from table.

12.32 P. M. Both pupils still very small; animal sits up.

1.15 P. M. Both pupils small, but definitely wider.

2.40 P. M. Pupils almost normal.

3.15 P. M. Both pupils about normal, respond to light.

*Experiment 2.* — Gray, young, female rabbit, 850 gm. Ether; cannula in vein.

11.50 A. M. Operation finished; ether discontinued.

12.00 M. Both pupils moderately dilated; started infusion of  $\text{CaCl}_2$   $m/8$ .

12.09 P. M. 12 c.c. in; both pupils definitely smaller.

12.13 P. M. 16 c.c. in; both pupils well contracted.

12.18 P. M. 21 c.c. in; stopped infusion; both pupils strongly contracted, but not pin point.

12.20 P. M. Wound sewed up and animal removed from board. Both pupils became wider than when on board; are still very contracted.

3.45 P. M. Both pupils practically normal size.

Although the weight of the animal in this last experiment was only a little above half the weight of the animal used in the other experiment, the effect of the calcium upon the pupil seemed to be stronger in the first than in the second animal. One of the reasons for this difference is to be found in the difference of the rate with which the infusion was permitted to go on in both animals. In the

first rabbit 21 c.c. of the solution was permitted to run in within ten minutes, while in the second rabbit about eighteen minutes were consumed for a similar quantity.

The following experiment demonstrates the dependence of the calcium effect upon the relation between the weight of the animal and the infused quantity of the solution.

*Experiment 3.* — Gray, female rabbit, 2650 gm.; cannula in anterior branch of external jugular vein; required very little ether.

10.10 A. M. Operation finished and ether discontinued.

10.30 A. M. Both pupils normal size; started infusion of  $\text{CaCl}_2$ .

10.42 A. M. 20 c.c. in; pupils smaller than normal.

10.48 A. M. 30 c.c. in; pupils getting smaller rapidly.

10.51 A. M. 35 c.c. in; stopped infusion; both pupils nearly pin point.

10.55 A. M. Wound sewed up, and animal removed from holder to the table; both pupils become slightly wider.

11.00 A. M. Pupils still well contracted; animal sits up, able to move about.

11.45 A. M. Both pupils almost normal size again.

In this experiment the rate of infusion was fairly rapid, 35 c.c. in twenty-one minutes. The weight of the animal, however, was more than one kilo greater than the weight of the animal in the first experiment. It had to receive therefore 35 c.c. of the calcium solution before the pupils became nearly pin point, and even then after the discontinuation of the infusion the pupils returned to their normal size in a much shorter time than that observed in the first experiment.

**The calcium effect upon the pupillomotor fibres of the sympathetic nerve.**  
— As mentioned before, we have in this investigation included the study of the effect of intravenous infusion of calcium on the pupillomotor action of the sympathetic nerve. One or both of the cervical sympathetic nerves were stimulated by induction currents of various strengths before, during, and after the infusion of the calcium solution, and the reaction of the pupils noted. In most cases the sympathetics were not cut or ligated centrally to the point of stimulation. Before stating the results of the effect of the infusion we should record very briefly one or two facts relating to the effects of stimulation of the normal (uninfluenced) sympathetic nerves. In the first place we should mention that the responses of

both nerves to stimulation run by no means parallel; that is, the same degree of current may often cause a different degree of reaction of the pupils to the stimulation of their respective nerves. In fact, we were sometimes confronted with animals in which either the right or the left sympathetic nerve would not react to any electrical stimulation. With regard to the vasomotor effect upon the blood vessels of the rabbit's ear S. J. Meltzer and Clara Meltzer<sup>2</sup> stated that "stimulation of the left sympathetic gave a distinctly better effect than stimulation of the right." The studies of the effect of stimulation of these nerves upon the pupils do not show such a preference for the left sympathetic nerve, at least as far as our present merely incidental observations show.

Another point worth mentioning refers to the effect of ether. While the animal was under the influence of ether, stimulation of the sympathetic nerves had frequently no effect whatsoever upon the pupil. This loss of irritability of the sympathetic nerves persisted in a variable degree for some time after the etherization was discontinued. As a rule, some time was permitted to pass after the etherization before the infusion of the calcium solution was started. In one or two cases after a few cubic centimetres of the solution had run in, the irritability of the sympathetic seemed to have improved and gave rise to an erroneous impression that calcium in small doses favors the irritability. However, such a favorable effect never occurred when a sufficient time was permitted to elapse after the stoppage of the etherization before the calcium infusion was begun.

Turning now to the relation of the calcium infusion to the effect of stimulation of the sympathetic nerves, we may state that, as a rule, under the influence of the calcium chloride the mydriatic effect of the stimulation of the sympathetic becomes greatly reduced. The reducing effect rarely becomes perceptible before 10 or 12 c.c. of the solution have run in. With the further infusion, however, in order to obtain a dilating effect the secondary coil of the apparatus has to be brought nearer and nearer to the primary coil, until 25 or 30 c.c. are run in, when even such a strong current as is obtained with a coil-distance of only 30 mm. produces no effect. In our preliminary communication we were inclined to assume that the irritability of the sympathetic nerves succumbs more readily

<sup>2</sup> S. J. MELTZER and CLARA MELTZER: *This journal*, 1903, ix, p. 66.

to the influence of the calcium than does the natural width of the pupil, that is, that the loss of irritability of the sympathetic may become manifest before the pupil shows a reduction in size. While such incidents have indeed occurred once or twice, an analysis of our entire material convinced us that such an unqualified statement is not well founded. On the contrary, there have been also instances in which the pupils were already considerably contracted, while the dilating effect of stimulation of the sympathetics was not yet strikingly reduced. Like the reduction in the size of the pupil, the reduction in the mydriatic effect of stimulation of the sympathetics depends upon the rate of flow of the infusion and of course also upon the relations between the weight of the animal and the injected quantity of the solution. The return of the irritability of the sympathetic occurs also in the same slow manner as was described for the return of the normal width of the pupil: it depends also upon the injected quantity of the calcium solution and the rate of injection. While in general it must be stated that there was a close parallelism between both kinds of effects of the calcium infusion, there were enough differences to justify the assumption that both reductions are due to different effects. We shall illustrate the chief points of the foregoing statement by the following abbreviated protocols of two experiments.

*Experiment 4.* — Gray female rabbit, 1695 gm. Ether; both cervical sympathetics isolated, intact; cannula in jugular vein. (Petzold coil, one Daniell cell.)

10.45 A. M. Operation finished; stopped ether.

11.00 A. M. Both sympathetics stimulated with 150 coil distance = good dilatation.

11.10 A. M. Both pupils moderately dilated; started infusion of  $\text{CaCl}_2$ .

11.18 A. M. 15 c.c. ran in; both pupils a little smaller than before; sympathetic with 150 c. d. = good dilatation.

11.22 A. M. 22 c.c. ran in; both pupils very small; both sympathetics with 90 c. d. = no response.

11.25 A. M. 30 c.c. ran in; stopped infusion; both pupils practically pin point; both sympathetics with 90 c. d. = no reaction.

(The experiment was continued with the intravenous injection of cocain.)

*Experiment 5.* — White male rabbit, 1907 gm. Ether; both sympathetic nerves isolated, intact; cannula in jugular vein.

- 3.36 P. M. Operation finished; stopped ether.  
 3.52 P. M. Both pupils moderately dilated; both sympathetics stimulated with 150 c. d.=good dilatation of pupils, left more prompt.  
 3.57 P. M. Started infusion of  $\text{CaCl}_2$  *m*/8.  
 4.05 P. M. 16 c.c. in; both pupils distinctly smaller.  
 4.07 P. M. 22 c.c. in; both sympathetics with 90 c. d.=no response.  
 4.09 P. M. Stopped infusion, 26.5 c.c. ran in; both pupils very small almost pin point; both sympathetics with 30 c. d.=no effect.  
 (Experiment continued with intravenous injection of cocain.)

**The influence of ether upon calcium myosis.**— In studying the causes for some of the variations in the intensity of the calcium myosis we found that the degree of etherization of the animal has a marked influence on the development and reversion of the calcium myosis. When the infusion began while the animal was still under the influence of ether, the myosis would develop slowly and might not even attain a strong degree. This would come out more strikingly when the etherization was continued during the entire infusion. Under the influence of ether the myosis would also disappear more rapidly after the infusion had been discontinued. The following two experiments will illustrate some of the points mentioned:

*Experiment 6.* — White female rabbit, 2110 gm. *Ether given heavily*; canula in jugular vein; both sympathetics exposed, intact.

- 11.55 A. M. Operation finished; *ether continued*.  
 12.00 M. Left and right sympathetics 70=0.  
 12.13 P. M. Ether relaxed slightly; left sympathetic 70=0, 50=strong dilatation; right 150, 130, 100=prompt dilatation; ether pushed again.  
 12.16 P. M. Started infusion of  $\text{CaCl}_2$  *m*/8.  
 12.23 P. M. 13 c.c. in; both pupils slightly smaller, still moderately dilated.  
 12.28 P. M. 22 c.c. in; both pupils as before, no increase in myosis; ether continued; lid reflex present, but reduced.  
 12.33 P. M. 29 c.c. in. Right sympathetic 100=0; 50=dilated, left sympathetic 50=0; 20=dilated.  
 12.34 P. M. 30 c.c. in; stopped infusion; both pupils moderately dilated; stopped ether, wound sewed up.  
 12.40 P. M. Both pupils respond to light, moderately dilated in shade.

*Experiment 7.* — Gray male rabbit, 1310 gm.; no ether; rabbit tied on Cannon board; both pupils moderately dilated.

- 12.20 P. M. Injected through ear vein about 23 c.c. of  $\text{CaCl}_2$  *m*/8; *both pupils become pin point*.

12.22 P. M. Injected into each of the hind legs, subcutaneously, 4 c.c. ether; animal removed from board.

12.24 P. M. Both pupils wider, but still well contracted; animal unable to get up.

12.25 P. M. Pupils more than  $\frac{1}{2}$  normal size, left wider than right.

12.30 P. M. (ten minutes after injection of  $\text{CaCl}_2$ ). Both pupils normal size, right pupil (faces window) slightly narrower; respiration good; lid reflex slight.

12.38 P. M. Animal dead; respiration stopped before heart.

**The action of mydriatics upon the calcium effect.**— We have tested the influence of mydriatics with relation to the above-described effect of calcium upon the pupil and the pupillomotor fibres of the cervical sympathetic. There are three different kinds of mydriatics, each of which exerts its influence through a special part of the pupillomotor mechanism. It is now generally accepted that atropin causes dilatation of the pupil by paralyzing the endings of the oculomotor nerve in the constrictor of the pupil; that cocain causes dilatation by stimulating the nerve endings of the sympathetic in the dilator muscle. Finally, that adrenin (adrenalin) causes mydriasis by stimulating the muscle of the dilator. These three different substances were tested by intravenous injection as well as by instillation into the conjunctival sacs. These substances were administered in one set of experiments shortly before the calcium infusion for the purpose of studying their influence upon the development of the calcium phenomena; in another set they were administered shortly after the phenomena made their definite appearance in order to study the disappearance of the latter under the influence of these mydriatics. The influence of adrenalin had to be studied in animals which were specially prepared for this purpose, *i. e.*, one of the superior cervical ganglia had to be removed at least twenty-four hours previous to the experiment. The adrenalin observations therefore were carried out in a separate set of experiments. The experiments with atropin and cocain, especially the instillations into the conjunctival sacs, were often carried out on the same animal, using one eye for one substance and the second eye for the other substance. We shall therefore describe our results with atropin and cocain practically together, using the same experiment for illustration of the results obtained with both of these substances.

**Atropin.**— Only very few experiments were made with intravenous injection of atropin; it caused no change whatever in the calcium effect. Instillation of atropin in the conjunctival sac *after* the calcium myosis was once established was never capable of changing the further course in any perceptible degree. When the pupils began to dilate, there was no difference between the two pupils; the atropinized pupil was not wider than its mate, which was used as control. Neither did it seem to accelerate the recovery of the irritability of the corresponding sympathetic nerve. Instillation of atropin *before* the beginning of the calcium infusion exerted undoubtedly some restraining effect upon the development of the calcium myosis. The pupil does not become as constricted as with calcium infusion alone, and even large doses of calcium did not under these circumstances bring the pupil to a maximal constriction. The reduction of the irritability of the sympathetic was also diminished, although here the neutralizing effect of the atropin was apparently less than on the calcium myosis. There were atropin experiments in which a fairly large dose of calcium finally reduced the irritability of the sympathetics to the same degree as with calcium alone, while the pupil remained only moderately constricted.

**Cocain.**— *After* the calcium effect upon pupils and sympathetic were well established, intravenous injections of 5 or 6 mgm. of cocain (which exerted an insignificant and only temporary effect upon the animal (twitching, restlessness, etc.), the irritability of the sympathetic showed a definite gain; strengths of currents which a few minutes before were without any effect showed now a definite dilatation. The irritability, however, remained far behind its original degree, that is, the degree which was established before the calcium infusion began. The myosis, however, is very little affected by cocain injections: the return to normal seemed to take the same course as without cocain injection. However, after each stimulation of the corresponding sympathetic the pupil retains its width unusually long.

When cocain was injected intravenously *before* the calcium infusion, the myosis which followed was moderately but definitely restrained. The reducing effect upon the irritability of the sympathetics apparently suffered also some restraint; this influence, however, was less manifest than in the cases where the cocain was injected *after* the infusion.

Instillation of cocain into the conjunctival sac *before* the infusion

of calcium had a strikingly neutralizing effect upon the action of the latter. In some instances the infusion of even twice the effective dose of calcium did not produce a characteristic myosis. In fact, in one or two cases the pupil remained as wide as normal and even slightly wider, although the pupil was never as wide as after instillation of cocain alone. While there was no doubt that calcium constricts even a cocainized pupil, the constriction was not comparable with that which was observed in a non-cocainized pupil. The calcium effect upon the stimulation of the sympathetic was also definitely reduced on the cocainized side, although this reduction seemed to be of a distinctly less degree than the one observed with relation to the myosis.

Instillation of cocain after the calcium myosis had developed had also a distinctly neutralizing effect, although perhaps not so striking as when instilled before the infusion. (For the effect of this instillation upon the stimulation of the sympathetic we find that our experiments permit no definite conclusions.)

The following experiments will illustrate some of the statements made with reference to the neutralizing actions of atropin and cocain:

*Experiment 8.* — Gray female rabbit, 1695 gm. Ether; cannula in vein; both sympathetics isolated, intact.

10.45 A. M. Operation finished; stopped ether.

11.00 A. M. Both sympathetics 150 c. d. = good dilation.

11.10 A. M. Started infusion of  $\text{CaCl}_2$   $m/8$ .

11.26 A. M. 30 c.c. in; stopped  $\text{CaCl}_2$ ; both pupils practically pin points; both sympathetics 50 = 0.

11.28 A. M. 5 mg. cocain injected into jugular, washed down with 2 c.c. saline; very slight twitching of legs and ears.

11.29 A. M. Very slight widening of the pupils.

11.31 A. M. Both sympathetics 50 = fair dilatation; pupils remain wider than before.

*Experiment 9.* — Black male rabbit, 1525 gm.; ether; cannula in jugular vein.

1.16 P. M. Started  $\text{CaCl}_2$   $m/8$ .

1.28 P. M. 21 c.c. in; stopped infusion; both pupils very small; instilled 2 per cent cocain in left eye only; right eye control.

1.35 P. M. Wound sutured, removed from board; left pupil a little wider now, right as before.

1.38 P. M. Instilled cocain again into left eye.

1.55 P. M. Right pupil very small; left pupil about four times larger than right.

2.00 P. M. Instilled a few drops of cocain again into left eye.

2.11 P. M. Right pupil same widening, but still well contracted; left pupil well dilated, *wider than normal*.

*Experiment 10.* — White female rabbit, 1750 gm. Ether; cannula in jugular vein; both sympathetics isolated, intact.

10.45 A. M. Instilled cocain (2 per cent) into left eye and atropin sulphate (1 per cent) into right eye.

10.55 and 11.13. Instilled again as before.

11.20 A. M. Cocain pupil now a little larger than atropin pupil. Left sympathetic 120 = good dilatation; right sympathetic 120 = same additional dilatation.

11.22 A. M. Started infusion of  $\text{CaCl}_2$   $m/8$ .

11.27 A. M. 9 c.c. in; cocain pupil much wider than atropin pupil.

11.29 A. M. 13 c.c. in; both sympathetics 120 = dilatation right better than before.

11.34 A. M. 30 c.c. in; both sympathetics 120 = dilating slowly.

11.36 A. M. 34 c.c. in; atropin pupil smaller than before; cocain pupil strongly dilated.

11.38 A. M. 37 c.c. in; left sympathetic (cocain) 120 = dilatation; right (atropin) 120 = 0, 70 = slight.

11.44 A. M. 45 c.c. in; cocain pupil much wider than atropin pupil.

11.52 A. M. 60 c.c. in; right (atropin) pupil half the size of left; left sympathetic 120 = dilates well; right sympathetic 50 = slight dilatation after same stimulation.

*Experiment 11.* — White male rabbit, 1885 gm. Ether; cannula in vein; both sympathetics isolated, intact.

10.55 A. M. Both sympathetics 150 = good dilatation.

11.02 and 11.10 A. M. Instilled cocain in left and atropin in right eye.

11.41 A. M. Atropin pupil well dilated; cocain pupil only moderately dilated, responds well to light; both sympathetics 150 = dilatation, better on cocain side.

11.44 A. M. Started  $\text{CaCl}_2$   $m/8$ .

11.49 A. M. 14 c.c. in; cocain pupil wider now than atropin eye.

11.57 A. M. 34 c.c. in; both sympathetics 90 = dilate only slightly.

12.00 M. 40 c.c. in; cocain pupil three times as large as the atropin pupil.

12.07 P. M. 53 c.c. in; both sympathetics 90 = slight dilatation, left sluggish.

12.10 P. M. 59 c.c.; right pupil smaller than before, left pupil a little wider than normal.

12.15 P. M. 68 c.c.; both sympathetics 50 = 0; right pupil (atropin) small (not as small as with Ca alone); left pupil (cocain) *a little wider than normal*, much larger than right pupil.

**The relation of the superior cervical ganglion and adrenalin to the calcium myosis.** — In 1898 Lewandowsky<sup>3</sup> observed that an intravenous injection of suprarenal extract produced a dilatation of the pupil, the maximum of which lasted only a fraction of a minute. This effect was well pronounced in cats and less definite in rabbits. This observation was soon confirmed by Boruttau,<sup>4</sup> Langley,<sup>5</sup> and others. It was further established that subcutaneous injection of the extract or its instillation fails to act on the pupil. In 1903 S. J. Meltzer and Clara Meltzer (Auer)<sup>6</sup> have found that, twenty-four hours after removal of the corresponding superior cervical ganglion, subcutaneous injections as well as instillations of the extract (adrenalin) cause a maximum dilatation of the pupil even in rabbits, and that the dilatation produced by these methods as well as by intravenous injection persists for many hours. Meltzer and Auer<sup>7</sup> have shown later that intramuscular injections of adrenalin work nearly as rapidly as intravenous injections. Lewandowsky and Langley, as well as the Meltzers, assumed that the dilatation which is produced by the extract is caused by the stimulation of the muscle substance of the dilator pupillæ. This assumption is now generally accepted. The Meltzers have further assumed that normally the superior cervical ganglion sends inhibitory impulses to the dilator muscle, which thus greatly restricts the efficient stimulation of that muscle by the suprarenal extract. This theory now also meets with an extended approval.

In our present investigations the questions arose: What influence upon the development of the calcium myosis would be exerted: (1) by the simple removal of the superior cervical ganglion; (2) by the injection or instillation of adrenalin with the ganglion present; and (3) by the injection or instillation of adrenalin twenty-four hours

<sup>3</sup> LEWANDOWSKY: Archiv für Physiologie, 1899, p. 360.

<sup>4</sup> BORUTTAU: PFLUEGER'S Archiv, 1899, lxxxviii, p. 112.

<sup>5</sup> LANGLEY: Journal of physiology, 1901-1902, xxvii, p. 237.

<sup>6</sup> CLARA MELTZER and S. J. MELTZER: Proceedings of the Society of Experimental Biology and Medicine, Feb. 28, 1903, xiii; Centralblatt für Physiologie, 1903, xvii, p. 651; This journal, 1904, xi, p. 28.

<sup>7</sup> S. J. MELTZER and J. AUER: Journal of experimental medicine, 1905, vii, p. 59.

and longer after the cervical ganglion has been removed? The experiments which were made to answer the above questions brought out, briefly stated, the following results:

1. The removal of a superior cervical ganglion does not restrain the development of calcium myosis. On the contrary, it seemed that in the absence of the ganglion the myosis attained its maximum with a smaller dose of the calcium solution.

2. Intravenous injection of adrenalin in normal animals (ganglion present) does not interfere with the calcium effect, either upon the width of the pupil or the irritability of the sympathetic nerve, whether the injection is administered before or after the efficient calcium infusion.

3. Intravenous or intramuscular injections of adrenalin before the calcium infusion in animals freed from one ganglion causes, upon the side where the ganglion was removed, *during* the infusion, a very moderate restraint, if any, upon the development of the myosis. After the discontinuation of the infusion, however, the pupil on the ganglion-free side dilates distinctly more rapidly than the pupil on the normal side.

4. Intravenous injection of adrenalin after the calcium infusion in a ganglion-free rabbit causes a marked hastening of the dilatation of the pupil on the ganglion-free side.

5. Instillation of adrenalin before the calcium infusion exerts very little restraint upon the development of the calcium myosis in the pupil of the ganglion-free side. They hasten somewhat the dilatation of that pupil after the discontinuation of the effusion, but this effect is much less than that observed after the intravenous injection of adrenalin.

(The observations on instillations of adrenalin after the calcium infusion do not permit any general conclusions.)

We append here a few abbreviated protocols of experiments which will illustrate some of the points mentioned.

*Experiment 12.* — Gray female rabbit, 1425 gm. Right superior cervical ganglion removed four days ago; ether; cannula in vein; left sympathetic nerve isolated.

10.05 A. M. Intramuscular injection of adrenalin; soon right pupil strongly dilated, left pupil moderately contracted.

10.16 A. M. Stopped ether.

10.30 A. M. Left sympathetic 120 c. d. = pupil dilates.

10.36 A. M. Started infusion of  $\text{CaCl}_2$   $m/8$ ; right pupil well dilated, left moderately dilated.

10.47 A. M. 20 c.c. in; both pupils contracted, left smaller than right.

10.50 A. M. 24 c.c. in; stopped calcium infusion; left sympathetic 70, 50 c. d. = slight.

10.55 A. M. Removed from board: left pupil practically pin point, right contracted but far from pin point.

11.55\* A. M. Right pupil moderately dilated, left pupil still strongly contracted.

12.20 P. M. Right pupil well dilated, left still strongly contracted.

*Experiment 13.* — Red-gray male rabbit, 1280 gm. Left superior cervical ganglion removed twenty-four hours before; ether cannula in vein; right sympathetic isolated, intact.

2.28 P. M. Operation finished; stopped ether.

2.52 P. M. Right sympathetic 150 = prompt good dilatation.

2.53 P. M. Started  $\text{CaCl}_2$   $m/8$ .

3.02 P. M.  $16\frac{1}{2}$  c.c. in; both pupils practically pin point.

3.04 P. M. 19 c.c. in; stopped infusion.

3.09 P. M. Injected 0.4 c.c. adrenalin into jugular vein, washed down with 2 c.c. saline.

3.15 P. M. Left pupil moderately dilated, right pin point.

4.45 P. M. Left pupil well dilated, larger than normal; right pupil wider than before, but still well contracted.

*Experiment 14.* — White male rabbit, 1720 gm. Left superior cervical ganglion removed eleven days before; ether; cannula in jugular vein; right sympathetic exposed, intact.

10.10 A. M. Operation finished; stopped ether.

10.15 A. M. Right sympathetic 130 = good, prompt dilatation.

10.41 A. M. Started  $\text{CaCl}_2$   $m/8$ .

10.45 A. M. 7 c.c. in; both pupils smaller, about equal.

10.48 A. M. 14 c.c. in; both pupils still smaller, left distinctly smaller.

10.50 A. M. 19 c.c. in; both very small, left smaller.

10.51 A. M. 21 c.c. in; stopped infusion; right sympathetic 100 = 0.

10.52 A. M. 0.6 c.c. adrenalin into jugular vein, 2 c.c. saline.

10.55 A. M. Left pupil dilated slightly, right pupil seemed to get smaller.

10.56 A. M. Right sympathetic 150, 100 = 0.

11.12 A. M. No change in right, very small, almost pin point; left pupil about twice the size of right.

11.22 A. M. Right pupil same, almost pin point; left pupil four to five times the size of right.

11.55 A. M. Left pupil almost normal in size; right pupil practically pin point.

12.05 P. M. Left pupil still wider, *wider than normal*; right pupil very small, no change.

1.10 P. M. Left pupil well dilated, more than before; right pupil very small.

1.55 P. M. As before; left pupil does not respond to light.

3.35 P. M. As before; right pupil practically pin point still; left pupil moderately dilated, responds slightly to light.

*Experiment 15.* — Gray female rabbit, 1220 gm. Right superior cervical ganglion removed twenty-one days before.

10.15, 10.40, and 10.50. Instilled adrenalin in each eye.

11.10 A. M. Right pupil dilated ad maximum, left apparently larger than normal.

11.45 A. M. Injected slowly through ear vein 35 c.c.  $\text{CaCl}_2$   $m/8$ ; both pupils strongly contracted; left pupil almost pin point, right a trifle wider than left.

12.30 P. M. Both pupils very small, about equal, and quite pin point.

2.10 P. M. Both pupils have widened; right larger than left and larger than before instillation; left a trifle less than normal.

**Asphyxia.** — As is well known, the normal pupils dilate during asphyxia, the dilatation as a rule being maximal or nearly so. In our investigation of the calcium myosis we have not made a direct study of the behavior of this myosis under the influence of asphyxia. We have, however, in the course of the present series of experiments, noted a number of cases in which one or the other animal suffered temporarily from or died under symptoms of asphyxia (convulsions, pulmonary œdema, etc.). By collecting and analyzing these incidental observations we are enabled to offer the following statement relative to the influence of asphyxia upon calcium myosis. When the infusion was still in progress and the myosis already well established, a temporary asphyxia due to a convulsion or a terminal asphyxia due to impending death caused only a slight widening of the pupil. The same was the case when asphyxia occurred after the discontinuation of the infusion, but only when the myosis showed a strong or fair tendency to persistence. There was, for instance, not a single case of strong or even fair dilatation of the pupil by asphyxia within an hour after the infusion when the animal received atropin either before or after the infusion of calcium. However, if the myotic pupil manifested already a

definite tendency towards dilatation, asphyxia did not fail to call forth a distinct additional widening of the pupil. This was especially manifest in cocain or adrenalin experiments, when the pupil began to dilate spontaneously. In other words, the mydriatic factor of asphyxia was of little account in antagonizing the calcium myosis when the intensity of the latter was not yet reduced. However, it became effective when the myosis was already otherwise weakened, or was originally only of moderate intensity. We have to add, however, that cases of myosis which were temporarily attacked by asphyxia have shown a tendency to an early return to normal, even if at the time of the attack the effect of asphyxia was very little manifest. The mydriatic action of asphyxia was therefore practically never completely lost in the process of antagonizing the calcium myosis.

**The effect upon the palpebral aperture.**— We have yet to record the fact that, besides the effect upon the pupil, the infusion of the calcium had a definite effect upon the tonus of the lids. In all cases when the infusion of calcium produced a definite myosis, the lids became (and remained) widely separate. Whereas at the beginning of the infusion closure of the lids often hampered the proper observation of the pupils, there was no such difficulty during the further progress of the infusion: the palpebral aperture was wide open and was rarely interrupted by spontaneous winking, although the lid reflex remained active. The same holds true also for the third lid; when the myosis was advanced, the retraction of the third lid was complete.

#### DISCUSSION. •

Before we enter into a discussion of the meaning of our observations, we shall first recapitulate briefly the main facts which our investigations brought to light.

An intravenous infusion of an M/8 solution of  $\text{CaCl}_2$  produces a very strong myosis, and abolishes or greatly reduces the irritability of the pupillomotor fibres of the sympathetic nerves. Asphyxia neutralizes the myosis only when it is already otherwise perceptibly weakened. Ether seems to neutralize the myosis to a somewhat greater degree than asphyxia. Atropin antagonizes the calcium effect only by instillation and altogether only to a very moderate extent. Cocain neutralizes the calcium effect better than any other mydriatic, also chiefly by instillation. It retards greatly the develop-

ment of the myosis, restrains the depression of the irritability of the sympathetic, and hastens the reversion of both after the discontinuation of the calcium infusion. The neutralizing effect of adrenalin upon the myosis of a pupil, the corresponding superior cervical ganglion of which was removed, stands between that of cocain and atropin, but it acts best through intravenous injection.

What is the cause of the calcium myosis, that is, which part of the pupillomotor mechanism is affected by calcium and in which manner? The normal pupil can be made to contract either through a stimulation of the constricting part of the apparatus or through an inhibition of the dilating part of it. There is normally a dilating tonus of the mechanism; this is demonstrated by the fact that cutting of the cervical sympathetic causes a constriction of the pupil. There might be some reasons for an assumption that the calcium influence in the production of the contraction of the pupil is of an inhibitory character. The contractions of a frog muscle after being immersed in a solution of sodium chloride are inhibited through the addition of some calcium chloride (Ringer, J. Loeb). Furthermore, according to J. B. McCallum, calcium inhibits the peristaltic movements of the intestines, that is, the movements of an apparatus in which smooth muscle and sympathetic nerve fibres are parts of its motor mechanism, and therefore in a way similar to the motor mechanism of the iris. However, it seems quite certain that the inhibition of the dilating tonus cannot be the cause of the calcium myosis, at least not its essential cause. The myosis following the calcium infusion is incomparably greater than the narrowing of the pupil which follows the section of the sympathetic or the removal of the superior cervical ganglion. There must be, therefore, an additional essential factor for the constriction of the pupil by calcium besides the inhibition of the dilating tonus. Besides, in some instances some time after the removal of the ganglion and probably also after complete degeneration of the sympathetic nerve endings, the corresponding pupil was often wider than on the other side. In these cases, without any nervous mechanism for a dilating tonus, the infusion of calcium did not fail to cause a strong myosis; *in fact, the effect seemed to appear more promptly in such cases.* It is therefore quite evident that it is in the constricting section of the pupillomotor mechanism where the cause for the calcium myosis must be sought, and that the action of the calcium must be of a stimulating and not of an inhibiting character.

In the constricting section of the mechanism we have to distinguish three parts, the stimulation of any one of which could cause myosis. The constriction could come through a stimulating action of the calcium either upon the substance of the constrictor muscle, or upon the endings of the short ciliary nerves within this muscle, or, finally, in any part of the nervous mechanism lying centrally to the nerve endings. Within the last-named part we could again distinguish nerve fibres, ciliary ganglion, and nerve centres lying centrally to the ganglion. But we can afford to omit discussing these details, as we can easily prove that none of the parts lying centrally to the nerve endings can be concerned in the calcium myosis, at least not to an essential degree. The proof is this. It is now well established that the mydriasis which is brought on by atropin is caused by a paralysis of the motor nerve endings of the constrictor muscle. We have stated above that atropin is capable of overcoming the calcium myosis only in a very small degree. However, such a myosis, which is caused by an action that is taking place centrally to the nerve endings, would be completely abolished by a paralysis of the nerve endings. The action of the calcium in producing myosis must therefore be either on the nerve endings or on the muscle itself.

There are, further, a number of facts which make it appear improbable that the myosis is chiefly due to a stimulation of the nerve endings. It is now fairly securely established that the myosis produced by physostigmin is caused by a stimulation of the nerve endings by that substance. If now atropin is instilled into an eye, the pupil of which is strongly contracted through a previous instillation of physostigmin, the pupil will become dilated nearly as much as if there had not been any previous myosis present. Here we see that atropin which paralyzes the nerve endings is capable of overcoming a strong stimulation of these organs by another substance. In our experiments we have found that atropin affects only slightly the calcium myosis. Now, if the myosis were caused only by a stimulation of the nerve endings, why should atropin not overcome it nearly completely? Furthermore, instillation or intravenous injections of adrenalin, which causes a maximal dilatation of the pupil, the corresponding superior cervical ganglion of which was previously removed, overcomes completely the myosis previously produced by physostigmin. This fact is well assured through statements in literature and through manifold experience of our own.

The effect of physostigmin is on the nerve endings of the constrictor muscle, that of adrenalin on the muscle substance of the dilator. Here we see that contraction of the dilator caused by a stimulation of its substance can completely overcome the contraction of the constrictor brought about by a stimulation of its nerve endings. The neutralization of the calcium myosis by the injection or instillation of adrenalin in gangliectomized rabbits is strikingly deficient. But if the calcium myosis were also due to a stimulation of the nerve endings of the constrictor, why should its neutralization not be complete or rather even reversed into mydriasis?

Here we may also refer to the fact that asphyxia and death overcome readily the myosis due to physostigmin, while they cause very little change upon the myosis brought about by calcium.

The various circumstances therefore seem to point inevitably to the conclusion that the myosis after infusion of calcium is brought about by a stimulation of the muscle of the constrictor pupillæ. We certainly can understand, then, why atropin has so little effect upon this myosis: the paralysis of nerve endings of the constrictor cannot interfere with a contraction which is caused by a stimulation of the muscle substance. However, if calcium stimulated the muscle alone, there would be no reason why atropin should then have even a slight effect upon the myosis produced by it, whereas our experiments demonstrated that, especially when atropin was instilled before the calcium infusion began, the development of the myosis was unquestionably interfered with. To meet this objection, the additional assumption has to be made that besides the muscle substance calcium stimulates also the nerve endings of the constrictor. The assumption which we offer reads, then, that *the myosis is produced by a simultaneous stimulation of nerve endings and muscle tissue of the constrictor of the pupil by the excess of calcium*. A substance like atropin, which promptly overcomes the stimulations of the nerve endings, would therefore reduce the degree of the myosis produced by calcium. It is possible that the calcium stimulation of the muscle tissue is more intense than that of the nerve endings. Hence the only moderate reduction of the calcium myosis by the instillation of atropin. However, it is not necessary to enter at present into a discussion of the merits of this special point.

There is nothing that we can think of which would militate against the assumption that besides the muscle tissue of the constrictor its nerve endings are also being stimulated by calcium.

On the other hand, this assumption will facilitate the understanding of the further fact that adrenalin which can overcome in the gangliectomized rabbit nearly completely the myosis produced by eserine should affect only moderately the myosis produced by calcium. The contraction of the dilator of the pupil brought about by a stimulation of only its muscle substance by adrenalin cannot completely overcome the strong contraction of the constrictor brought about by a simultaneous stimulation of the muscle and its nerve endings. On the same basis we can understand without a detailed argumentation why asphyxia which overcomes the myosis of eserine affects only moderately the calcium myosis.

With regard to the "nerve endings" of the sphincter we ought to refer to the distinction made by H. K. Anderson,<sup>8</sup> namely, that these "endings" consist of two parts: a more centrally located part which is the point of attack by eserine, and a peripherally located part of the "endings" which is stimulated by pilocarpin and paralyzed by atropine. Applying this subdivision to the subject under discussion, it may be assumed that it is only the peripheral part of the nerve endings which is stimulated by calcium simultaneously with the contractile substance. This would in a way be more acceptable, since this peripheral part is supposed to have closer affiliation with the muscular tissue. On the other hand, we would have to insist that it is not this part alone which is affected by calcium, since atropine paralyzes it, while atropine does not overcome the calcium myosis.

There is one fact among our observations which offers some difficulty in its explanation. It is the observation that cocaine neutralizes the calcium myosis better than atropine and better even than adrenalin. From the fact that atropine and adrenalin, which readily overcome the effects of eserine myosis, exert only a moderate effect upon the calcium myosis, the impression may be gained, at least in a general way, that the calcium myosis is more resistant than the myosis produced by eserine. But here we are confronted with the fact that cocaine, the mydriasis of which is readily overpowered by eserine, is more capable of antagonizing the calcium myosis than the more powerful mydriatics atropine and adrenalin, which would thus seem to indicate the reverse, namely, that the resistance of the calcium myosis is less strong than that produced by eserine. The

<sup>8</sup> H. K. ANDERSON: *Journal of physiology*, xxxii, *Proceedings of the Physiological Society*, 1905, xlix; 1905-1906, xxxiii, p. 414

comparison of the actions of cocain and adrenalin seems to be especially puzzling. Cocain is assumed to act by stimulation of the nerve endings and adrenalin by stimulation of the muscle of the dilator. Cocain neutralizes the calcium myosis a good deal better than adrenalin. Can we assume that a contraction of the dilator produced by stimulation of the nerve endings is stronger than one which is produced by the direct stimulation of the muscle? But we might indeed be inclined to answer this query in the affirmative, remembering the fact that for the frog muscle, in order to bring out a certain degree of contraction, the direct stimulation requires a stronger stimulus than the indirect. However, in the present fermenting state of our knowledge regarding the nature of "nerve endings," "myo-neural junction," "receptive substances," etc., we hardly know what is meant by the statement that adrenalin stimulates the muscle cells and cocain the nerve endings, and can therefore not discuss satisfactorily these differences.

We may add, further, that the experiments which led to the conclusion that cocain acts on the pupil by stimulation of the nerve endings of the dilator of the pupil have only considered and eliminated the possibility that the dilatation, which is brought on by cocain, is caused by a paralysis of the motor nerves of the constrictor. There is, however, another possibility which, as far as we know, has not yet been even considered. The assumption is entertained by many physiologists and ophthalmologists that the normal process of dilatation of the pupil is aided by an inhibition of the constrictor of the pupil, that is, that with each motor impulse to the dilator runs simultaneously an inhibitory impulse to the constrictor, thus causing a dilatation of the pupil without any wasteful struggle of the antagonistic muscles. May not the effect of cocain consist in a similar action, that is, in a simultaneous stimulation of the motor mechanism of the dilator and the inhibitory mechanism of the constrictor, — in other words, may not cocain stimulate not only the motor nerves of the dilator muscle, but *stimulate the entire mechanism of dilatation*? If this be the case, cocain would effect the neutralization of the calcium myosis by causing a contraction of the muscle dilator, inhibiting at the same time, at least in some degree, the contraction of the constrictor of the pupil. We could then readily understand why such an action should be more effective than the action of adrenalin, which causes only a contraction of the dilator muscle. However, we have for the present no new

facts in support of this hypothesis and shall not enter into a further discussion of its merits.

Our experiments brought out the further fact that under the influence of the infusion of calcium the cervical sympathetic gradually loses its dilating action upon the pupil. This might be interpreted in two ways. It might mean that under the influence of calcium the pupillomotor fibres of the sympathetic become paralyzed, which would mean that calcium exerts here an action entirely different from and independent of the one which causes myosis. But it could also mean that the impulses of the sympathetic to the dilator muscle, which even under the influence of calcium remain normal, are insufficient to cause a dilatation of the pupil on account of the inability of the dilator to overcome the strong contraction of its antagonist, the constrictor. We have already indicated at the outset that in general there was a certain parallelism between the development of the myosis and the reduction of the irritability of the sympathetic nerves. For instance, the quantity of calcium which brought out one phenomenon was generally sufficient to permit the appearance of the other phenomenon. This and other similar facts speak in favor of the assumption that there is a close interdependence of both phenomena. On the other hand, there were a number of instances in which the myosis remained practically unaffected while the sympathetic nerve manifestly regained its irritability to a considerable degree. While, on the basis of the general impression which we gained from our observations, we are rather inclined to accept the assumption that the loss of the irritability of the sympathetic nerves is indeed a phenomenon independent of the development of the calcium myosis, the meagreness and indefiniteness of the concerned facts prevent us at this stage of the work from going on record with such a positive statement. Future investigations may throw more light on that subject.

#### SUMMARY.

An intravenous infusion of an  $m/8$  solution of  $\text{CaCl}_2$  causes invariably a maximal contraction of the pupils. The myosis may be completely developed at a stage of the infusion when no other signs of a calcium intoxication are present. With the development of the myosis the pupil loses its responsiveness to light and to other

physiological myotic or mydriatic reactions. The myosis is apparently due essentially to a stimulation of the muscle of the constrictor of the pupil. It is probable that also the motor nerve endings are stimulated to some-degree by the calcium. (Perhaps only the mural part of the nerve endings (H. K. Anderson) is affected.)

Parallel to the development of the myosis the palpebral aperture becomes stationary and wider, and the "winking" becomes rare, although the lid reflex proper may still be entirely unaffected.

The infusion of calcium reduces also greatly the irritability of the pupillomotor fibres of the cervical sympathetic nerves. It is fairly probable that the loss of irritability of the sympathetic is an independent phenomenon and not simply a consequence of the strong myosis.

After discontinuation of the infusion the calcium effects may continue in their maximal degree for an hour and longer, and many hours may pass before the pupils return to their normal condition.

Atropin neutralizes the myosis only to a very small degree, and this only by instillations carried out before the beginning of the calcium infusion.

Cocain, by instillations as well as by intravenous injections, exerts a much more evident neutralizing action upon the calcium effects than atropin. It retards their development and accelerates their disappearance. However, even the neutralizing action of cocain must be designated as only moderate.

Intravenous or intramuscular injections or instillations of adrenalin exert also a definite neutralizing action upon the calcium effects in gangliectomized animals. The degree of the action stands between that of atropin and cocain.

Ether frequently retards the development and hastens the disappearance of the calcium myosis.

Asphyxia overcomes only to a very limited degree a well-developed calcium myosis; it favors, however, its early disappearance.



**The Present Status of the Serum  
Therapy of Epidemic Cerebro-  
Spinal Meningitis**

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**SIMON FLEXNER, M.D.  
NEW YORK**



# THE PRESENT STATUS OF THE SERUM THERAPY OF EPIDEMIC CEREBRO- SPINAL MENINGITIS \*

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NEW YORK

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My intention is to present a very brief statement of the status of the serum treatment of epidemic meningitis. It is now about three years since the serum was first used therapeutically in this country. Gradually its employment has extended until now it is being used in Great Britain, France and Germany, as well as in the United States and Canada. Indeed, the Rockefeller Institute has sent supplies to India and to Jerusalem, but, as no reports have been received from these distant places, they will not be further considered at present.

At the time of the first employment of the serum in this country the epidemic of meningitis had already receded in the East, and, although the disease spread westward even beyond the Rocky Mountains and was often very severe and fatal, yet the number of cases arising or being recognized in any one community was not large. I, therefore, view the test made of the serum in America as having coincided with the period of recession of the epidemic.

At the period of the first employment of our serum in Belfast and Edinburgh the epidemic was at its height: that is to say, it had reached its maximum development. from which it was tending to recede rather than to extend. All the evidence at hand shows that the disease at that time had not diminished in virulence and fatality, but the number of cases appearing was in a given time less than before.

In Germany, also, the epidemic was virtually at an end when the serum was received. In France, on the

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\* Read in the Section on Practice of Medicine of the American Medical Association, at the Sixtieth Annual Session, held at Atlantic City, June, 1909.

other hand, the serum was available at the beginning of the outbreak of the epidemic which is now prevailing in Paris and the provinces. To the fortunate circumstance that Professor Calmette, on his return from the International Congress on Tuberculosis, carried with him to Lille a considerable quantity of the serum is to be ascribed its prompt employment. Subsequently the Rockefeller Institute sent large supplies to him, to Professor Netter and to Professor Roux. The reports of the serum treatment now appearing in French medical journals are based chiefly on the employment of the serum prepared at the Rockefeller Institute.

If, therefore, the decision of the value of the serum treatment was properly withheld until the opportunity arose to subject it to a test at the beginning of a severe epidemic, when the fatality is commonly at its height, this opportunity has now arrived in France. The outlook is further promising for a comparative study of cases of epidemic meningitis treated with the serum and in other ways. While the serum is being employed widely, apparently, in Paris and in the intense way that experience has indicated to be the best, namely, by successive injections of relatively large doses, in the provinces it is being less generally employed and it has been found difficult to have the intense method carried out by the provincial practitioners. When the reports are all in and the figures have been collected we may expect, therefore, valuable information on the value of the serum.

In the meantime, I can report to you that the excellent results obtained from the serum in America and Great Britain are being repeated in France. There have already been published reports covering 100 or more cases of the disease treated with the serum in which the mortality will probably be less than 25 per cent. On the other hand, a smaller number of cases thus far reported treated by other methods have given a far greater mortality and one approaching 80 per cent. Moreover, the phenomena of diminution in severity of the symptoms and reduction of the period of infection and convalescence have been observed there as elsewhere.

We may, then, consider fairly, I think, that the serum treatment has now been subjected to test under a variety of conditions, some of which were as severe as probably ever occur. And yet I should still advise caution in concluding that the case has been proved for the serum.

The total number of reports of cases of epidemic meningitis treated with the serum prepared at the Rockefeller Institute which I have collected is under one thousand, and it obviously will take a larger number than that to establish its value. I wish now to present a tabulation which has recently been prepared based on 712 cases of the disease in which the bacteriologic diagnosis was made and the serum treatment used. In the first table the cases are subdivided according to certain age periods, and in the second the total cases of each age period are further subdivided according as the serum was injected in the three arbitrarily chosen periods of duration of the disease.

TABLE 1.—CASES OF EPIDEMIC CEREBROSPINAL MENINGITIS TREATED WITH THE ANTIMENINGITIS SERUM

CASES ANALYZED ACCORDING TO AGE GROUPS

Age years.	Total no. cases.	Recovered.	Died.	% Mortality.
1-2	104	60	44	42.3
2-5	112	82	30	26.7
5-10	113	95	18	15.9
10-15	101	73	28	27.7
15-20	107	72	35	32.7
20+	175	106	69	39.4
Total, all ages	712	488	224	31.4

Table 1 brings out several points of interest. The highest mortality is shown to have occurred in the first two years of life. But contrary to the rule under the older forms of treatment in which the mortality was 90 per cent., or over, in this series it was 42.3 per cent. The second age period is from 2 to 5 years, in which the mortality was 26.7 per cent. The third age period embraces children from 5 to 10 years of age and gave the lowest mortality of all, namely, 15.9 per cent. The next period extends from 10 to 15 years and gave a mortality of 27.7 per cent. The next period of from 15 to 20 years showed a considerable rise in mortality, equaling 32.7 per cent., and the last period, embracing the cases of 20 years and over, gave a mortality of 39.4 per cent. The average mortality in all the age periods was 31.4 per cent.

The time allowed me is insufficient to enable me to enter into a detailed discussion of the figures. It is my intention, however, to consider them in some detail in the near future. A point of importance and one to which Dr. Jobling and I have already referred is the importance of experience with the serum in securing the best results from it. If the separate large series of cases treated by individual observers are analyzed, the great

discrepancy in the results as between children over 2 years of age, young adults and adults past 20 years are not encountered.

TABLE 2.—CASES OF EPIDEMIC CEREBROSPINAL MENINGITIS TREATED WITH THE ANTIMENINGITIS SERUM  
CASES ANALYZED ACCORDING TO DAY OF INJECTION

Ages. Yrs.	Period of Injection—Day.								
	1st-3rd			4th-7th			Later than 7th		
	Rec.	Died.	%	Rec.	Died.	%	Rec.	Died.	%
1-2	16	1	5.8	22	10	31.2	22	33	60.
2-5	24	6	20.	40	12	23.	18	12	40.
5-10	43	8	15.6	35	6	14.6	17	4	19.
10-15	36	8	19.	23	9	28.1	14	11	40.
15-20	25	17	40.4	25	8	24.2	22	10	31.2
20+	36	21	36.8	34	24	41.3	36	24	40.
Totals..	180	61	25.3	170	69	27.8	129	94	42.1

Table 2 is instructive in bringing out the importance of early injections of the serum. The results in the first two years of life are especially noteworthy. The extraordinary figures given under the first period of injection, namely, in the first three days of the disease, can hardly be maintained. But the influence of period of injection is shown by the rapid rise in mortality in the subsequent two periods. The rule of the effects of early injection is preserved in the age periods up to the period of from 15 to 20 years, when it disappears. The discrepancy occurring in the two highest age periods cannot be wholly accounted for at present. The explanations which suggest themselves are that among older individuals there tends to be a large number of very severe, rapidly fatal or fulminating cases of the disease, or that older persons are less subject to the beneficial action of the serum. As regards the actual proposition, it may be stated that adults not infrequently respond promptly to the serum injections by abrupt termination of the disease or ameliorated symptoms and pathologic conditions.

The total figures do not, however, fail to indicate that the early injections are more effective than the later ones, as is shown by the percentage mortality in the first-to-third-day period of 25.3, in the fourth-to-seventh-day period of 27.8, and the period later than the seventh day of 42.1.

There is one consideration which I should like to touch on. The study of certain groups of cases of epidemic meningitis, coming under one observer, indicates that the diagnosis can sometimes be made before the usual symptoms of meningeal irritation appear or are recognizable. The cerebrospinal fluid removed by exploratory lumbar puncture has been, in these cases,

sometimes clear and sometimes turbid and contained more or less polynuclear leucocytes and always *Diplococcus intracellularis*. The serum being injected immediately, these cases almost invariably were abruptly terminated or ran relatively a mild course. Attention having been drawn to the cases as being possibly examples of epidemic meningitis because of the similarity of the prodromal symptoms to those of other cases diagnosed later after the signs of meningeal irritation were plain, they ran a short and mild course after the early administration of the serum, while the others, which served to direct the attention, having been injected later when the infection and inflammation were well established, assumed a far more severe and protracted form.

Rockefeller Institute for Medical Research.

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## THE DESTRUCTIVE EFFECT OF SHAKING UPON THE PROTEOLYTIC FERMENTS.

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### INTRODUCTION.

IN brief preliminary communications<sup>1</sup> we have already stated that shaking exerts a destructive influence upon the proteolytic enzymes. Since these reports were made the experiments were gone over critically again and were also extended. We intend to give in the present paper in the first place a detailed account of the methods which were employed and the facts which were found in the course of this investigation. We wish, however, to deal in this paper with the subject of shaking from a wider, a more general point of view. In the chemical studies of the action of ferments, a field which in recent years is being so extensively cultivated, the shaking of digestive mixtures for many hours in succession belongs to the routine procedures. If shaking produces such a profound effect upon enzymes as was found in our experiments, it certainly must be a factor in obtaining the results of these chemical studies. Nevertheless it is only very recently that some physiological chemists came out with observations of this kind. We shall discuss later these observations in connection with our own experience. We wish to say here, however, that it was not an accidental stumbling upon such a fact that aroused our attention. Our starting-point was the accumulated evidence regarding the effect of shaking upon corpuscular elements of microscopical dimensions. One of us (Meltzer) has been interested in this latter problem for the last twenty-five years. In a paper by this author, published in 1894, the subject of the "Importance of vibration to living matter"

<sup>1</sup> SHAKLEE and MELTZER: Zentralblatt für Physiologie, 1909, xxiii, p. 3; Proceedings of the Society for Experimental Biology and Medicine, vi, pp. 48 and 103.

was discussed at some length. Based upon evidence derived from experiments of his own and upon an analysis of the data collected from the literature, the writer arrived at the conclusion that vibration is an important factor in the processes of life. It was on the basis of this that we began to study the problem of the influence of shaking upon enzymes. Since the publication of the mentioned paper other interesting investigations appeared on the subject of shaking upon corpuscular living bodies. The results obtained in these new studies are in harmony with the previously expressed views.

In discussing later the nature of the processes which take place in the inactivation or destruction of ferments by shaking we shall try to gain a point of view from which we could interpret the action of shaking in an identical manner for the enzymes and the corpuscular bodies. It is therefore desirable to have here also an account of what is known of the action of shaking upon these latter elements. We shall therefore preface the report upon our experiments by a brief review of the literature upon the subject of the influence of shaking upon corpuscular elements.

#### THE INFLUENCE OF SHAKING UPON LIVING BODIES OF MICROSCOPICAL DIMENSIONS.

Horvath<sup>2</sup> — 1878 — was the first to study the influence of shaking upon microorganisms; he found that by a certain method of shaking bacteria their growth may be retarded, or they may even become completely destroyed. Although Naegeli<sup>3</sup> considered this statement to be of considerable importance to biology, practically no serious study of that subject followed Horvath's investigation. Among the dozen or more writers whom Meltzer could quote in 1894 on the subject of shaking, there seemed to be a complete disagreement regarding the effect of shaking. Some stated that it is destructive, some that it is without any effect, and some even maintained that shaking exerts a beneficial effect. The trouble with these investigations was that in each case another organism was tested, and by another method. Moreover, none of the investigators used the method which was employed by Horvath. The

<sup>2</sup> HORVATH: *Archiv für die gesammte Physiologie*, 1878, xvii, p. 125.

<sup>3</sup> NAEGELI: *Theorie der Gährung*, München, 1879.

objects which were tested in these investigations were mostly mixtures of bacteria; in a few instances the bacteria were definite species, and in two instances definite species of yeasts were shaken.

Independent of the experiments of Horvath, Meltzer and Welch <sup>4</sup> — 1884 — studied the influence of shaking upon the red cells of bullock's blood. Accidentally they used a method of shaking similar to that which was employed by Horvath. In addition to that, Meltzer and Welch increased the effect of shaking by adding granular substances to the dilute blood. It was found that the red corpuscles became completely destroyed by shaking. Of the particulars only a few points should be mentioned here. The destruction was the more rapid, the heavier the added granular substances and the finer they were. No fragments of the corpuscles could be observed; the red cells were converted into "dust."

In the investigations reported by Meltzer in his paper of 1894 <sup>5</sup> the effect of shaking was studied upon well-defined bacteria, and the results were studied by the usual bacteriological methods. Glass beads were added to the bacterial suspensions; otherwise the method was the same as the one employed for the red blood corpuscles. The results differed now greatly with the species of bacteria, with the duration of the shaking and with the energy with which the shaking was carried out. There were bacteria for which the average moderate degree of shaking was destructive; others for which the very same shaking was beneficial. One species was found which practically was not growing at all without a certain degree of shaking; it grew best at a higher degree of shaking, and only at a very strong degree of shaking it became destroyed. The destruction of bacteria consisted, like that of the red blood corpuscles, in the conversion into "dust"; the shaking never led to a breaking down into fragments.

On the basis of his experiments and of those of others Meltzer has put forward the supposition that shaking or vibration is one of the physiological factors of life; a minimum of shaking is indispensable to the living organism; there is an optimum degree at which the organism thrives best and there is a maximum degree of shaking beyond which it is destructive to the life of the organism. Minimum, optimum, and maximum, however, vary with each

<sup>4</sup> MELTZER and WELCH: *Journal of physiology*, 1884, v, p. 255.

<sup>5</sup> MELTZER: *Zeitschrift für Biologie*, 1894, xxx, p. 464.

species of organism. Some algæ grow only under waterfalls and some bacteria become destroyed merely when their cultures are carried from one room to another.

In 1900 Meltzer<sup>6</sup> studied again the influence of shaking upon red blood corpuscles. This time blood cells of various animals were shaken, and in many experiments glass beads were added. Here are some of the main results: The simple act of defibrination shortens already the life of the corpuscles. Shaking with glass beads is capable of completely destroying the corpuscles of all animals. The time required for destruction differs with the species of animals. For the blood cells of some animals (guinea pigs) a certain small degree of shaking proved to be beneficial, — it prolonged their life. Again, as in the previous experiments with Welch, the corpuscles broke down to "dust," never into fragments. When the shaking was continued, the dust became beaten together to little clumps or to ragged threads, which grew in size with the continuation of the shaking. Hæmoglobin crystals did not break down by shaking. Shaking therefore appeared to affect only *organized*, so to say, living minute bodies, converting them into "dust"; it does not break down crystals or unorganized minute organic masses.

Of special interest are the observations made on the influence of shaking upon certain echinoderm eggs. Morgan<sup>7</sup> observed that shaking hastens the maturation of starfish eggs, it accelerates the extrusion of the polar bodies and causes the disappearance of the nuclear membranes. Eggs which have been shaken before fertilization develop in much larger numbers than eggs which were not shaken. Mathews<sup>8</sup> made the further interesting observation that starfish eggs might develop without fertilization — artificial parthenogenesis — by shaking them (after maturation) in a test tube or by squirting them from a syringe. Too strong shaking of the eggs causes a dissolution of the whole egg. Similar observations were made by Fischer<sup>9</sup> on the eggs of amphitrites. The amount of agitation necessary to bring about a parthenogenetic development of the eggs of susceptible species varies with the individuals of the same species. On the other hand it was established by

<sup>6</sup> MELTZER: Johns Hopkins Hospital reports, 1900, ix, p. 135 (Contributions to the science of medicine, dedicated to William Henry Welch).

<sup>7</sup> MORGAN: Anatomischer Anzeiger, 1893, p. 141.

<sup>8</sup> MATHEWS: This journal, 1902, vi, p. 142.

<sup>9</sup> FISCHER: This journal, 1902, vii, p. 303.

Mathews and by Loeb that eggs of *Arbacia* cannot be made to develop parthenogenetically by any degree of shaking. Mathews and Whitcher,<sup>10</sup> as well as Meltzer,<sup>11</sup> found that strong agitation may cause a destruction of the unfertilized as well as of the fertilized *Arbacia* eggs. They also stated that shaking may hasten the course of development of the fertilized eggs. This, however, was not confirmed by Whitney.<sup>12</sup> The above-mentioned authors have also found that fertilized eggs of *Arbacia* are much more resistant to the destructive effect of shaking than the unfertilized eggs. According to Meltzer the unfertilized eggs are converted by violent shaking into "dust-like debris," while the fertilized eggs show only disorganized eggs and coarse fragments.

We may point out that the interesting experiments upon echinoderm eggs bring out again the fact that shaking may be harmful as well as favorable, depending upon the species of the animal and to a degree even upon the individual from which the eggs were obtained, and also according to the degree of shaking which was employed.

From this brief review we learn that in the past thirty years the physiological influence of shaking was the subject of many investigations. The subjects of these experiments may be roughly divided into three groups, — bacteria including yeast, echinoderm eggs, and red blood corpuscles. Bacteria and eggs are cells, living organisms.

Red blood corpuscles differ from the other cells essentially by the lack of power of reproduction. Nevertheless they are usually considered as living cells. They perform a vital function, they are subject to metabolic processes and they die. Red blood corpuscles are certainly organized units of living matter.

We may point out here again that in the second series of experiments of Meltzer upon red blood corpuscles it was clearly demonstrated that clumps of organic matter are affected by vigorous shaking in an entirely different manner from organized living cells (red blood corpuscles).

From the various investigations we have learned that organized living bodies of microscopical size are profoundly affected by shaking. With some degree of shaking and with some species of these organized bodies the influence may be a favorable one: bacterial

<sup>10</sup> MATHEWS and WHITCHER: This journal, 1903, viii, p. 300.

<sup>11</sup> MELTZER: This journal, 1903, ix, p. 245.

<sup>12</sup> WHITNEY: Journal of experimental zoölogy, 1906, iii, p. 41.

cultures may grow more rapidly, blood cells may live longer, and some echinoderm eggs may develop even parthenogenetically. Vigorous shaking causes a destruction of these organisms; but the destruction again is specific for the organized living bodies; they do not break down into fragments or coalesce into formless masses; red blood corpuscles and bacteria are "converted into dust" and some echinoderm eggs undergo a complete "dissolution."

These facts entitle us to look upon shaking as one of the influential conditions of life; it is with shaking as it is with heat, some degrees are indispensable, others present an optimum, and still others act as a destructive maximum, and these degrees vary with the species of the living organisms.

As to the nature of the mechanism through which shaking produces such effects as are mentioned above, we may restate here briefly the theory offered by Meltzer in the above-mentioned paper on the "Importance of vibration to living matter." In living organized matter the physical molecules are, according to this theory, collected into groups, *physiological units* (in contradistinction to physical units), which are completely separated from one another by a system of connected spaces carrying liquid. These units are in continued vibration, which keeps the liquid in the drainage system in continual motion. By this motion oxygen and other necessities are carried to, and waste products are removed from, these units by the way of the drainage system. In cells and in other seemingly homogeneous living organized matter the metabolic exchange is carried on, perhaps not by simple diffusion, but *by a circulatory system in which the vibrations of the physiological units replace the pumping action of the heart and the system of spaces represents the vessels.* The physiological units are set into motion by vibrations received from outside. Therefore a certain degree of shaking or vibration is indispensable for all living matter. It is obvious that some degree of shaking may be an optimum, while a violent shaking may disrupt all living matter, converting it into the dust of the living units. In higher animals the shocks coming from the heart beats, the respiration, etc., might be sufficient to provide the most distant cells with the necessary vibration. Organisms which do not have such an aid within themselves receive these vibrations from outside and thrive only where they can be provided with this factor of life. It is easily comprehensible that according to the grouping of the physiological units and their relation to their in-

ternal system of drainage the various organisms might differ greatly with regard to the degree of vibration they require — some algæ get what they need only under a waterfall.

It was on the basis of these facts and views that we approached the question: Can shaking influence ferments? We do not know the "substance" of ferments, we know these only by their action. We are willing to admit that ferments are not living organisms. But they are invariably associated with them, exert a great immediate influence upon life phenomena, are activated and destroyed by most factors which favor and destroy life. May we not assume that the carriers of ferments, while deprived of many characteristics of living cells, may show nevertheless that organization of living matter which was assumed above for living organisms, namely, that also the structure of the carriers of enzymes consists in vibrating physiological units separated from one another by a system of liquid-carrying spaces? These were the considerations which started us upon the present investigation, the details of which we are now going to report.

## OUR EXPERIMENTS.

**Methods of shaking.** — On the basis of the foregoing statement we may expect that the vibrations within each class of living matter are somewhat specific in their nature, and that therefore a shaking which should be effective ought to be of a specific kind, capable in each case to produce adequate vibrations. This holds good especially for vibrations which are capable of producing favorable results. For the destructive effects we may perhaps assume that the shaking need not be of a very specific kind. However, from the very first experience in that line, from the experiments of Horvath, we learned that even for the purpose of destruction the shaking must be carried out in a definite way. Horvath obtained no results when he attempted to shake the mixtures of bacteria by a rotating apparatus or by a swinging pendulum. His positive results were obtained only when the bacteria were shaken by a machine which shook the bottles horizontally in the direction of their long axis. We have already stated that few investigators have taken the precaution to use Horvath's method in repeating his experiments. Some who have seen no effects of shaking were using rotating machines, or the bottles within a machine were struck so and so many times a minute (B. Schmidt, Whitney, and others). In the second series of experiments by Meltzer with red blood corpuscles it was established that there is a striking difference in the effect whether

the bottles were shaken in the direction of their long or their transverse axis.

In the present investigation the shaking was carried out in the manner used by Horvath and employed also by Meltzer in his various investigations on the effects of shaking. Long, round bottles were employed which had a capacity of 115 c.c., a length (neck not included) of 14 cm. and a transverse diameter of 3.6 cm., and were charged usually with 10 c.c. of the solutions to be shaken. The bottles were securely placed in the carriage of a shaking machine, the movements of which were in the direction of the long axis of the bottles. The length of the excursions was about 8 cm. The rate of the movements in one direction varied between 100 and 150 times in a minute. Two shaking machines were employed; one is kept in the basement of the building where the temperature during the period of experimentation varied between 13° and 23° C.; the other is kept in a thermostat in which the temperature is kept approximately at 33° C. Controls were kept in the neighborhood of each machine under the same conditions except for the shaking.

The effect of shaking was studied in this series as stated at the outset upon the three proteolytic enzymes: pepsin, trypsin, and renin. Of these pepsin was studied first and more extensively than the others.

**Newer methods of testing for pepsin.**—The activity of this ferment was measured by three methods which were introduced very recently especially for clinical purposes: the Jacoby-Solmes<sup>13</sup> ricin method, the edestin method of Fuld,<sup>14</sup> and the casein method of Gross.<sup>15</sup> The principle of the Jacoby method consists in the fact that ricin (impure) dissolved in a neutral salt is precipitated by HCl. The heavy milky precipitate is digested by pepsin. The Fuld method rests upon the fact that neutral salts bring out a precipitate in a solution of edestin (edestin in HCl), but not in that of its digestion products. The method of Gross is based upon a similar principle: acetic acid causes a precipitation in a solution of casein, but not in that of caseoses.

The particulars of these methods, as they are described in the medical literature, were worked out chiefly with an eye upon their use for clinical purposes. In this investigation details of the quantitative tests were slightly modified, especially in the methods of Fuld and of Gross. At the beginning of the research the ricin method of Jacoby was employed exclusively. In the further course, however, the methods of Gross and of

<sup>13</sup> (JACOBY-)SOLMS: *Zeitschrift für klinische Medizin*, 1907, lxiv, p. 159.

<sup>14</sup> FULD: after Wolff and Tomaszewsky, *Berliner klinische Wochenschrift*, 1907, p. 1051.

<sup>15</sup> GROSS: *Berliner klinische Wochenschrift*, 1908, p. 643.

Fuld were also employed, especially the latter, which offers some advantages over the other methods.

The particulars of the ricin method, as it was employed here, were as follows: Into each of a series of test tubes 0.05 c.c. of a pepsin solution was carefully measured from a pipette graduated to hundredths. Some of the tubes received shaken solutions, others received the corresponding controls. Then into each tube was run 0.5 c.c. of a solution of HCl (0.5 per cent). Finally 2 c.c. of a ricin solution (1 per cent ricin in a 5 per cent aqueous solution of NaCl) were quickly run into each tube from a 10 c.c. pipette. One or two tubes were prepared without pepsin for the purposes of comparison later in estimating the amount of digestion. The tubes were now corked and the contents thoroughly mixed by inverting the tubes a sufficient number of times. All tubes were then placed in a thermostat, kept at 37° to 38° C., and examined about every twenty or thirty minutes; deductions were derived from a comparison of the amount of the precipitate at each examination as well as from a comparison of the times (Schütze's law) when complete digestion took place.

The Fuld method was used in a modified form, as appears from the following description: To find the amount of pepsin destroyed by shaking, we determined how much of the shaken pepsin solution was necessary to digest 2 c.c. of a 0.1 per cent edestin solution,<sup>18</sup> in the same time as a given quantity of the unshaken solution (control). It was thought desirable to make the digestion period about two hours in order to reduce the error due to the digestion that would take place in the first tubes of the series between the time when the edestin solution was added to them, and when it was added to the succeeding tubes. It was found that if the 1 per cent solution of pepsin which was the one we usually used were diluted 25 times, 0.5 c.c. of this dilution would about digest 2 c.c. of the edestin solution in two hours; hence 0.5 c.c. of the control solution was used as the standard, and the shaken solution was so diluted, when possible, as to require about 0.5 c.c. of the dilution to digest 2 c.c. of edestin solution in the same time. The test was carried out in the following way: The standard quantity of pepsin was run into each of the first two of a series of test tubes, from a pipette graduated to hundredths of 1 c.c., then increasing quantities of the diluted shaken solution were run into the succeeding tubes. Next the volumes in all the tubes were made the same by adding dilute HCl of the same strength as that employed in making the pepsin solutions and their dilutions. Finally 2 c.c. of a 0.1 per cent solution of edestin in a 0.1 per cent aqueous solution of HCl was rapidly run from a 10 c.c. pipette into each tube. The tubes were then corked and the contents thoroughly

<sup>18</sup> For the edestin we have to thank Dr. P. A. Levene.

mixed. They were kept in the thermostat at 40° C. for about two hours. After removal from the thermostat the tubes were placed in the refrigerator to cool, and after they had cooled, a saturated solution of NaCl was carefully run down the sides of the tubes, until it formed a considerable layer underneath the digestion mixture. The tubes were then allowed to stand for fifteen to twenty minutes. At the end of this time each *standard* tube had a faint white ring between the salt solution and digestion mixture, while the *comparison* tubes showed a gradation of rings ranging in density from lighter than the standard to heavier. The comparison tube which had a ring like the rings of the standard tubes was regarded as having the same quantity of pepsin in it as the standard quantity, and the difference between this quantity and the amount it contained before shaking was regarded as the quantity of pepsin destroyed by the shaking; *e. g.*, if the quantity of shaken solution in the comparison tube represented 0.10 gm. pepsin in the solution before shaking, and the standard quantity of pepsin were 0.02 gm., it was concluded that 80 per cent of the pepsin activity had been destroyed by the shaking. (The results seem to be most accurate when the rings are examined in a suitable light against a suitable background. In most tests the rings were illuminated by direct sunlight and examined against a dark background.)

The Gross method was modified in much the same way as the Fuld method. After the tubes had been charged with the pepsin solutions and the volumes made equal as there described, 2 c.c. of a 0.1 per cent solution of casein in dilute HCl was quickly run into each tube from a pipette, and the contents mixed and digested as with the Fuld method. After digestion the end point was found by using a saturated solution of sodium chloride in a 5 per cent aqueous solution of acetic acid, in the manner in which we used the saturated aqueous solution of NaCl in the Fuld method. The amount of pepsin destroyed by shaking was calculated in the way there described.

In most experiments commercial pepsin (Fairchild) was used in 1 per cent solution; in only a small number of experiments the solutions were of 0.1 per cent or of other strengths. Again in most experiments pepsin was dissolved in dilute HCl (0.25 per cent); in some experiments only 0.1 per cent or other concentrations of HCl were used; in some no HCl was used.

Other variations of the method will be mentioned when describing the results.

#### THE INFLUENCE OF SHAKING UPON PEPSIN.

It may be stated at the outset that every one of the experiments brought definite evidence that shaking, as we employed it, is

capable of exerting a destructive influence upon the activity of pepsin. The following experiment will illustrate this statement:

*Experiment 1.* — Five bottles, each were charged with 10 c.c. of a solution of pepsin (1 per cent + 0.5 per cent HCl). The space above the solution in bottles Nos. 76 and 81 was filled with hydrogen, in bottles Nos. 75, 79, and 80 the space was filled with air. Bottles Nos. 75 and 76 were shaken continually for eight hours in the thermostat at 33.7° C. at the rate of 104 per minute. Bottles Nos. 79, 80, and 81 were kept also in thermostat, but not shaken (controls). Five test tubes were then prepared as follows: To two of the test tubes 0.05 c.c. of the shaken pepsin (one with air and one with H), and to each of the other 3 test tubes 0.05 of the unshaken pepsin was added. Then 0.5 c.c. of HCl 0.5 per cent was run into each tube, and finally 2 c.c. of ricin solution. All test tubes were now incubated at 38° C. for digestion. The result is shown in Table I.

The result is unmistakable. Whereas the unshaken pepsin was sufficient in quantity and quality to digest the heavy precipitate of the ricin within one hour, the same quantity of the same pepsin, but shaken for eight hours at 33.7° C., did not affect the precipitate even after keeping the tubes for four days at a digestion temperature.

In one of the shaken tubes in these experiments the space above the pepsin solution was filled up with hydrogen. This was done to demonstrate that the destruction was not due to the intimate mixing of the pepsin solution with the oxygen of the air, perhaps a sort of detrimental oxidation. In a number of experiments the space above the pepsin solution was filled with hydrogen or carbonic acid gas. There was no difference in the result whether that space was filled with air or with the mentioned gases. We shall cite an experiment in which one of the bottles was filled with CO<sub>2</sub>.

*Experiment 2.* — Four bottles each received 15 c.c. pepsin solution prepared as stated above. The spaces above the solution in bottles 10 and 12 contained air, in bottles Nos. 11 and 13 CO<sub>2</sub> gas. Bottles 10 and 11 were shaken one hour in the thermostat at 34° C.; bottles 12 and 13 were kept also in the thermostat, but unshaken (control). Four test tubes were then prepared as follows: Into each tube was run 0.05 c.c. of pepsin solution (one charge from each bottle), then HCl and ricin as in the previous experiment. The 4 test tubes were then incubated in the thermostat (38° C.) for digestion. Table II. shows the result.

TABLE I  
RICIN TEST FOR PEPSIN

Incubation time began at 10 A. M., Oct. 12, 1908.	QUANTITY DIGESTED.				
	Shaken 8 hours.		Controls.		
	No. 75.	No. 76.	No. 79.	No. 80.	No. 81.
10.32 A. M., Oct. 12, 1908 . .	0	0	per cent. 90	per cent. 85	per cent. 85
11.00 A. M., Oct. 12, 1908 . .	0	0	Complete	Complete	Complete
10.25 P. M., Oct. 12, 1908 . .	0	0	.....	.....	.....
8.24 A. M. Oct. 13, 1908 . .	0	0	.....	.....	.....
8.00 A. M., Oct. 13, 1908 . .	0	0	.....	.....	.....
8.00 A. M., Oct. 14, 1908 . .	0	0	.....	.....	.....
5.45 P. M., Oct. 14, 1908 . .	0	0	.....	.....	.....
7.45 A. M., Oct. 15, 1908 . .	0	0	.....	.....	.....
7.45 P. M., Oct. 15, 1908 . .	0	0	.....	.....	.....
8.00 A. M., Oct. 16, 1908 . .	0	0	.....	.....	.....

This experiment shows in the first place that shaking even for one hour (at 34° C.) affects greatly the activity of the pepsin. While the unshaken pepsin digested nearly all of the precipitate in thirty-five minutes, it took the shaken pepsin about twenty-four hours to digest as much as the unshaken ferment digested in half an hour. The experiment shows further that in the presence of CO<sub>2</sub> (instead of air) the destruction of the pepsin goes on with at least the same effectiveness as in air. In fact, in the few experiments in which the pepsin was shaken for short periods in an atmosphere of CO<sub>2</sub> the destruction seemed to be even more advanced than when shaken for similar periods in an atmosphere of air. At any rate, it is quite evident that the destruction of the pepsin is not due to some process of oxidation.

**Influence of duration of shaking.**— From the difference in the results of the foregoing two experiments the fact can be derived that the degree of the destruction grows with the length of the

period of shaking. While in the first experiment, in the tube containing pepsin which was shaken for eight hours, no sign of digestion of the precipitate could be detected even after a continuous incubation in the thermostat for four days, we notice in the second experiment that in the tube containing pepsin which was shaken

TABLE II.  
RICIN TEST FOR PEPSIN.

Incubation time began at 9.00 A. M., Sept. 29, 1908.	QUANTITY OF PRECIPITATE DIGESTED.			
	Pepsin shaken one hour at 34°.		Controls.	
	No. 10 Air.	No. 11 CO <sub>2</sub> .	No. 12 Air.	No. 13 CO <sub>2</sub> .
9.35 A. M., Sept. 29 . . . . .	per cent. 0	per cent. 0	per cent. 95	per cent. 95
11.05 A. M., Sept. 29 . . . . .	50	50	Complete	Complete
3.50 P. M., Sept. 29 . . . . .	70	60	.....	.....
9.20 A. M., Sept. 30 . . . . .	90	70	.....	.....
12.20 P. M., Oct. 1 . . . . .	Complete	90	.....	.....

one hour only, about one half of the precipitate was digested two hours after incubation, and after forty-eight hours all, or nearly all, was digested. This point was studied directly by several series of experiments, of which the following is an illustration.

*Experiment 3.* — Twelve bottles, each containing 10 c.c. of pepsin solution No. 20 (1 per cent pepsin, 0.1 per cent HCl) with air in the space above, were shaken variable periods in thermostat at 33° C. at the rate of about 150 per minute. The pepsin was tested by the edestin method (Fuld). Table III. gives the percentages of destruction to the duration of shaking.

This and other similar series of experiments prove to a certainty that the duration of the shaking has a manifest influence upon the degree of destruction of the pepsin; the longer the shaking lasts, the more of the pepsin becomes destroyed. The destruction is, however, not directly proportional to the time of shaking. The

main destruction takes place within the first eighty minutes of shaking. What is left of the activity of the pepsin proves quite resistant and gives way very slowly to the destructive influence of the continued shaking.

**Influence of temperature.** — Of great influence upon the destructive effect of shaking is the temperature at which the shaking is

TABLE III

No. of bottle.	Duration of shaking in minutes.	Percentage of destruction.	No. of bottle.	Duration of shaking in minutes.	Percentage of destruction.
293	5	15+	300	60	80 ±
294	10	35+	301	82	90 ±
295	15	43	302	102	95 ±
296	20	45 ±	303	131	97 ±
297	30	50-60 ±	304	186	98 ±
298	40	55-56 ±	305	264	98.5 ±
299	50	74 ±	....	....	....

being carried on. As stated previously, shaking was carried on by us also in a machine which is located in the basement of the building, where the room temperature is generally lower than in the upper parts and is subject to the influence of the fluctuating external temperatures. The temperature surrounding this machine was sometimes as low as 13° C. and was rarely, if ever, higher than 23° C. The following table illustrates the influence of shaking at a temperature of 21° C.

TABLE IV.

**DESTRUCTION OF PEPSIN BY SHAKING AT 21° C., TESTED BY THE EDESTIN METHOD.**

Duration of shaking.	Percentage of destruction.
30 minutes . . . . .	22 per cent
90 minutes . . . . .	56 per cent
180 minutes . . . . .	62 per cent
372 minutes . . . . .	94 per cent
726 minutes . . . . .	99 per cent

Each of these figures was derived from several experiments, the results of which, however, varied but little in each case. For instance, the figure for the percentage of destruction by shaking one hundred and eighty minutes was derived from different analyses which gave the figures: 70, 65, 64, 60, 60, 60, 60, 60. A comparison with Table III will show the striking difference in the effect of shaking at a temperature lower by  $12^{\circ}$ . Shaking at  $33^{\circ}$  C. for thirty minutes destroyed at least 50 per cent, while the same duration of shaking at  $21^{\circ}$  C. destroyed only 22 per cent of the pepsin. The same difference holds good for longer periods of shaking. The importance of the duration of shaking is, at the lower temperature, even more manifest than at  $33^{\circ}$  C., and striking is here also the resistance of the undestroyed residuum of pepsin: after six hours' shaking the destruction was 94 per cent, and after twelve hours there was still at least 1 per cent pepsin left undestroyed.

**No reactivation.** — In a number of experiments after shaking for periods which usually produce more or less complete destruction, some of the bottles containing this shaken pepsin were kept in the refrigerator ( $5^{\circ}$  C.) and some in the thermostat ( $38^{\circ}$  C.) for various periods. It was found on testing even after six days that there was no sign of a recovery of the ferment activity.

**Presence of beads indifferent.** — In some experiments the bottles subjected to shaking contained a fair number of solid large beads. We could not find that the presence of the beads favored in a notable degree the destruction of the pepsin.

**No destruction in full bottles.** — In some other experiments all air was excluded, the shaken bottle being filled to the stopper with the pepsin solution. In these bottles there was hardly any destruction of the pepsin. Even after shaking continually seven days, the destruction was not more than 4 per cent, if so great, compared with that of the control. All of these bottles contained beads. There is very little shock communicated to the fluid when it cannot move within the bottle.

**Shaking in paraffined tubes.** — In one or two experiments the bottles to be shaken were paraffined inside before they received the pepsin solution. The destruction was practically the same as in non-paraffined bottles.

**Shaking in sealed tubes.** — In order to exclude the possible chemical effect of the rubber in a number of experiments the shaking was carried out in test tubes, the mouths of which were sealed on the

gas flame. An exact comparison of the results could not be made on account of the difference in the diameter and the volume of the bottles. However, the destruction of the pepsin in the sealed tubes was as prompt as in the bottles with the rubber stoppers.

**HCl a favorable factor.** — In the majority of the experiments, as stated above, hydrochloric acid was added to the pepsin, which acted at the same time as an antiseptic. In many experiments, however, toluol was added to the pepsin solution instead of HCl. (In control experiments it was first established that toluol alone hardly interferes with the action of pepsin.) The destructive effect of the shaking was manifest also in these experiments. It appeared, however, that the destruction was not as marked in these solutions as in those which contained HCl. In a few instances the pepsin solution contained only water. In these cases also the destruction was not as good as when the solution of pepsin contained HCl. From these facts it appears that the presence of HCl favors the destructive action of shaking.

**Influence of rate.** — We have not made systematic observations upon the influence of the rate of shaking upon the destruction. But an analysis of the data shows that the destruction was manifestly more rapid when the number of movements of the bottles per minute was greater.

**Addition of peptone.** — Only four experiments were made with the addition of 1 per cent peptone to the pepsin solution (with HCl). The destructive effect of shaking was strikingly reduced — destruction amounting to only 25 per cent or less in shaking for twenty hours at 33° C. Glycerine also appears to retard greatly the destructive effect of shaking. However, the number of these experiments is too small to permit at present a definite conclusion in this regard.

**Shaking gastric juice.** — In addition to shaking pure solutions of (commercial) pepsin in a number of experiments the gastric contents of a dog were shaken from two to twenty-four hours. The general result is that the pepsin in these contents becomes also destroyed by shaking. However, there are apparently a number of qualifying factors connected with the pepsin of the gastric juice of the dog's stomach which have not yet been studied. We shall therefore not enter into the particulars of these experiments.

**Shaking by respiratory movements.** — On the supposition made by Meltzer in the paper on the "Importance of vibration," that the

rhythmical shocks within the animal body, like those made by the cardiac and the respiratory movements, are capable of producing vibratory effects, the attempt was made to expose pepsin solutions to the effects of such movements. Two series of experiments were instituted to establish this purpose. In one series suitable bottles containing solutions of pepsin were introduced into the stomach of a dog through an œsophagus fistula. In another series such bottles were placed in the peritoneal cavity of rabbits. For the stomach experiments either small vials were used, closed by rubber stoppers and tightly secured by rubber dam against the entrance of gastric juice, or small glass tubes sealed on the gas flame; or the pepsin solution was placed in tightly closed rubber finger cots. In all cases the containers were kept within the stomach, secured by a cord attached to them by one end, while the other protruding end was tied around the neck of the animal. The bottles within the stomach did not inconvenience the animal, which partook of food in the usual manner. For the peritoneal cavity small bottles with stoppers or sealed glass tubes were used. They were introduced through a small opening which was immediately sutured. In either case the bottles remained in their respective places for various periods, in the case of the peritoneal cavity as long as seven days.

There was a definite destruction of the pepsin in practically all experiments. In the stomach the greatest reduction took place in the rubber finger cots. (They were used on the supposition that in these soft containers the pepsin solution might be subjected to the "massaging" effect of the movements of the diaphragm.) However, even here the reduction was not higher than 40 per cent compared with the activity of the pepsin in the control kept for similar periods in the thermostat at about 38° C.

In the bottles which were kept in the rabbit's abdomen the destruction of the pepsin was definitely more pronounced than in those kept in the dog's stomach. However, maximum thermometers, which were simultaneously kept within the abdomen, indicated that the temperature there, at least at times, must have been higher by a degree or two than the temperature in the rectum of the animal or in the thermostat in which the control was kept. Since it was found (Shaklee) that difference in temperature even only of a few degrees when lasting for days is capable of producing a palpable difference in destruction, it was difficult to ascertain how much of the observed destruction might have been due to the

action of the temperature. While we are thus not entitled on the basis of our present experiments to give definite data, we may be nevertheless justified in stating that in these experiments there was a degree of destruction above that which could be accounted for by the effects of the elevated temperature. Our provisional theory is that this part of the destruction is due to the shaking caused essentially by the respiratory movements.

### THE INFLUENCE OF SHAKING UPON TRYPSIN.

**Quantitative method.** — For the quantitative determination of trypsin the method of Gross<sup>17</sup> was used which is based upon the fact that acetic acid causes a turbidity in an alkaline casein solution, but not in the solution of the caseoses. In the method recommended by Gross the procedure is as follows: Into each of a series of test tubes, 10 c.c. of an alkaline casein solution is run (casein solution — 1 gm. of casein and one of  $\text{Na}_2\text{CO}_3$  to 1000 distilled water). To each of these tubes increasing quantities of the solution containing trypsin is added and the tubes incubated in a thermostat at 40° C. After fifteen minutes a few drops of acetic acid is added to each tube, which produces a turbidity in the tubes in which digestion is incomplete. The strength is calculated from the smallest quantity of the trypsin solution which prevented the appearance of turbidity.

A modification of this method was used in this research similar to that which was used in the determination of pepsin. To two of a series of test tubes, equal (standard) quantities of a standard solution of trypsin were added, and into the other tubes increasing quantities of a chosen dilution of the solution to be tested were run. The volumes were made equal, and then 2 c.c. of a neutral casein<sup>18</sup> solution were run into each. The tubes were incubated for an hour or two in the thermostat at 40° C. Then a saturated solution of NaCl in a 5 per cent aqueous solution of acetic acid was run down on the side of each tube and allowed to stand fifteen to twenty minutes. The rings which were formed in the test tubes containing the solution to be tested were compared with the rings of the standard tubes. The tube containing a ring similar to that of the standard tube was selected, and from the quantity of shaken trypsin of that tube compared with the quantity of unshaken in the standard tube, the strength of trypsin was calculated and expressed in per cent of the original strength.

<sup>17</sup> GROSS: *Archiv für experimentelle Pathologie und Pharmakologie*, 1907, lviii, p. 157.

<sup>18</sup> KUDO: *Biochemische Zeitschrift*, 1908, xv, p. 473.

Grübler's trypsin was used in 0.1 per cent solutions. The shaken solutions were usually alkaline, some containing toluol. In a few instances aqueous solutions were shaken.

**Results.** — The main result is here again that shaking exercises a destructive effect upon trypsin. We shall not enter here into many details. The following short table will illustrate the main points.

TABLE V.

SHOWING THE EFFECT OF VARIOUS DURATIONS OF SHAKING TRYPSIN (0.1 per cent)  
AT 21° C.

Duration of shaking.	Percentage of destruction.
30 minutes . . . . .	68 per cent
90 minutes . . . . .	83 per cent
186 minutes . . . . .	90 per cent

The figures given here are averages from several experiments in which each individual figure was lower or higher than the average by only 1 or 2 per cent.

We learn from this table that trypsin is readily destructible by shaking, that the destruction takes place in a very marked degree, even at such a low temperature as 21° C.; that the main destruction takes place within the first half-hour, and that the smaller the residuum of the trypsin is, the greater is its resistance to the destructive effect of shaking.

**Different tryptsins.** — With reference to the last-mentioned point we wish to say that according to Vernon<sup>19</sup> trypsin consists of several tryptsins which differ in their resistance to the destructive action of Na<sub>2</sub>CO<sub>3</sub>. We may therefore assume these various tryptsins differ perhaps also in their resistance to the destructive influence of shaking.

**Trypsin less resistant than pepsin.** — The table indicates also the interesting fact that trypsin is more readily destroyed by shaking than pepsin: more was destroyed of trypsin (0.1 per cent) in thirty minutes at 21° C. than of pepsin (1.0 per cent) by shaking forty minutes at 33° C. The difference between the resistance of the two proteolytic ferments was found to exist also when both were shaken in distilled water and in the same concentration.

<sup>19</sup> VERNON: *Journal of physiology*, 1900-1901, xxvi, p. 405.

## THE INFLUENCE OF SHAKING UPON RENIN.

**Method of testing renin.** — Pepsin solutions, neutralized to litmus, were used for the study of renin. To two of a series of tubes standard volumes of a standard solution of the ferment were added, and into the remaining tubes increasing quantities of a chosen dilution to be tested were run in. The volumes were made equal, and finally 5 c.c. of skimmed fresh milk (+ 0.4 per cent CaCl) was quickly run into each tube. The tubes were kept at room temperature for about fifty minutes. The action of the ferment was indicated by the appearance of an incipient coagulum on the side of the tube after tipping it. The measure of ferment strength was obtained by determining how much of the solution to be tested was necessary to produce the same coagulation effect as a given quantity of the standard solutions.

**Result.** — It may be briefly stated that renin was destroyed by shaking practically in the same degree as pepsin. Shaken solutions of pepsin have shown nearly the same destruction of their renin contents, when tested in neutral solution, as was found for the pepsin content, when tested in acid solution. All the various conditions which influenced the resistance of pepsin to the action of shaking acted in the same manner also on renin. For reasons which will be manifest later we should mention especially that the presence of HCl in the pepsin solution favored the destruction of renin.

**The rise of temperature by shaking.** — It has been urged by some writers that the effect of shaking might be due to a rise of temperature produced by the shaking. Although it is fairly obvious that the degree of destruction which we have observed in this research could not have been caused simply by the rise of temperature, we tried to establish by direct observation the rise of temperature which is produced by such shaking as we employed. Through a hole in the side of a bottle which contained pepsin solution and beads a maximum thermometer was tightly inserted so that the part containing mercury was bathed in the solution. The thermometer was thus placed at a right angle to the axis of the bottle so that the movements of the bottle could not affect the column of mercury. The bottle was wrapped in heavy paper. The result is a surprise: in five experiments the highest rise was less than 0.3° C.

Summing up our experimental results, we have to say that without laying too much weight upon the details which were ascertained or upon the exactness of the figures which were obtained in these researches, the gross results were of such a kind as to leave no doubt regarding the truth of the following facts: all three proteolytic ferments can become completely destroyed by shaking, at least by such shaking as was in use in this research. The destruction or inactivation was not reversed within six days, during which period some of the shaken ferment solutions were kept in the refrigerator and some in the thermostat. Higher temperatures ( $33^{\circ}$  C.) favor destruction by shaking. The duration of shaking is also an important factor in the action of shaking; the longer the ferment solution is shaken, the more of it becomes destroyed. On the other hand, the smaller the undestroyed residuum of the ferment becomes, the longer it takes to destroy it. There is a great deal of difference in the resistance to the destructive effect of shaking between pepsin and trypsin. The bulk of the latter is more readily destroyed and at lower temperatures than pepsin. However, an undestroyed small residuum of trypsin retains a remarkable resistance which is, perhaps, even greater than that of the residuum of pepsin. There is practically no difference in the effects of shaking between pepsin and renin. It is probable that the respiratory movements are capable of producing some degree of destruction upon the proteolytic ferments exposed to their action.

#### RESULTS OF OTHER INVESTIGATORS.

When we started this research, we were not aware of any investigation or statement bearing upon the possibility of a destructive effect of shaking upon the activity of ferments. However, soon after the appearance of our preliminary communication in the "Zentralblatt für Physiologie" Professor Abderhalden was kind enough to send us an article by himself and Guggenheim,<sup>20</sup> calling our attention to a passage in it which deals with the destructive influence of shaking upon solutions of tyrosinase. After shaking it for twenty-four hours at  $37^{\circ}$  C. it lost its activity greatly. It was strikingly inhibited also when shaken at room temperature.

<sup>20</sup> ABDERHALDEN and GUGGENHEIM: *Zeitschrift für physiologische Chemie*, 1908, liv, p. 352.

Similar experience they had with zymase: shaking forty-eight hours at room temperature retarded its fermentative activity considerably; when shaken for twenty-four hours in the incubation chamber, it became completely inactive. Experiences of that kind they had also with pancreatic juice, but they give no particulars.

In the course of this summer two more articles appeared which deal with the influence of shaking upon ferments. One concerns us directly, as it deals with the influence of shaking upon renin; it is by Signe and Sigval Schmidt-Nielsen.<sup>21</sup> They prepared the renin from the mucosa of the calf's stomach. Their shaking was done by an apparatus in which the bottle was fixed, but the solution within it was agitated by means of a perforated plate moving to and fro in the direction of the long axis of the bottle two hundred and fifty times per minute. The destructive effect upon the renin was judged by the time it required to cause coagulation in milk. Their report contains no statement showing a complete destruction of the renin by shaking. The longest duration of the shaking which is mentioned in their communication is only one hour. They obtained, however, already a considerable degree of "inactivation" of renin by shaking it only a few minutes, and this at a temperature of only 16° C. The inactivation was the more complete, the greater the rate of shaking, the longer it lasted and the higher the temperature was at which it was shaken. So far the statements of the Schmidt-Nielsens are essentially in agreement with ours. They state, however, that they could not obtain any results with commercial preparations of renin on account of the presence of HCl in these preparations, which in their experience prevents the "shaking-inactivation," as they term that phenomenon. In our experience rather the reverse was the case, the presence of HCl favored the destruction of renin as well as that of pepsin. However, we should not enter into a discussion of some minor discrepancies between the two investigations. The entire subject is new, and we welcome any statement which is in agreement with the fundamental facts in this research.

The second paper is by Harlow and Stiles,<sup>22</sup> on the effect of shaking upon the activity of ptyalin, and was induced by our preliminary communication. Shaking dilute saliva (1:10) in plain

<sup>21</sup> SIGNE and SIGVAL SCHMIDT-NIELSEN: *Zeitschrift für physiologische Chemie*, 1909, lx, p. 426.

<sup>22</sup> HARLOW and STILES: *Journal of biological chemistry*, 1909, vi, p. 359.

bottles reduced the digestive effect upon starch only moderately; the reduction was improved by the addition of glass beads. The greatest part of the reduction occurred during the first half hour, afterwards it proceeded in a diminished rate. By this method the ptyalin ferment was never reduced to more than half of its activity. The activity was further reduced by the introduction of new beads; but the authors never succeeded in rendering the ferment completely inactive.

Wisps of glass wool were more effective than glass beads. Unclean glass beads or glass beads heated to redness had no effect.

If we understand the description of the arrangement for shaking in the experiments of Harlow and Stiles, the bottles must have remained at all times in a vertical position and the liquid within the bottles could have received only moderate shocks. The effect of shaking by such a method is therefore not comparable with the action of such effective methods as were employed by us and the Schmidt-Nielsens, and probably also by Aberhalden and Guggenheim. The fact, however, that even by their moderate mode of shaking (and apparently at room temperature) Harlow and Stiles have evidently observed a definite reduction of the digestive power of the ptyalin ferment confirms the chief point of the contention that shaking is capable of reducing the activity of a ferment.

We have thus now definite data for the action of shaking upon several ferments. Aberhalden and Guggenheim established that shaking for twenty-four hours at the incubation temperature or twice as long at room temperature inhibits completely the fermentative effect of an oxydase and of zymase. We have shown that by our method of shaking the activity of all three proteolytic ferments can be completely abolished within a much shorter time. The Schmidt-Nielsens have found that by their method of shaking more than one half of the activity of renin can be destroyed in a few minutes. Harlow and Stiles observed a reduction of the activity of ptyalin by shaking. Furthermore, we have to mention that Aberhalden and Guggenheim state that they obtained similar results from shaking pancreatic juice. Probably the activity of all three ferments of that juice were inhibited by shaking. With regard to the action of shaking upon lipase, we have yet to mention that an abstract of a paper on "Human pancreatic juice" by Harold C. Bradley,<sup>28</sup> presented at the last meeting of the

<sup>28</sup> BRADLEY: *Journal of biological chemistry*, 1909, v p. 191.

American Society of Biological Chemists, contains the brief statement that "continuous shaking in a machine was found to inhibit the digestion (of fat) markedly."

Summing up, we may therefore state that as far as the digestive ferments are concerned, we have for each of the ferments evidences from two sources that shaking is capable of exerting a destructive influence upon their digestive activity. Taking further into consideration the observation of Abderhalden and Guggenheim that shaking can completely inhibit also a vegetable oxydase (tyrosinase) and of zymase (or only the proteolytic activity of the latter?), we are justified in making the general statement that a certain degree of shaking is capable of reducing or completely inhibiting the fermentative action of enzymes. Furthermore, since in our experiments pepsin and trypsin were subjected to exactly the same method of shaking, and in some experiments even all other conditions being exactly the same and nevertheless trypsin was distinctly more readily destroyed than pepsin, we may draw the further general conclusion that ferments differ in their resistance to the same degree of shaking.

As to the nature of the effect the Schmidt-Nielsens speak intentionally of "inactivation," meaning hereby that the effect is only a temporary one and is reversible. Abderhalden and Guggenheim avoid the term injury and prefer to say that the ferment becomes inactive or that the activity becomes inhibited. We are speaking in this paper of the destruction of the ferments. The existence of the ferments is known only by their activity. In our experiments we have seen the complete disappearance of these activities and have not seen any sign of a return of these activities, even after keeping the shaken solutions of these enzymes in the incubator for a good many days. As to the statement of the Schmidt-Nielsens that in their experiments "die Schüttel-Inaktivierung unter gewissen Umständen einen gewissen reversiblen Prozess darzustellen scheint," we cannot discuss it properly until they have stated under which conditions the process "seems" to be reversible. But we wish to say this: even if we assume that by a certain degree of shaking the ferments are irrevocably destroyed, we may well admit that preceding this final state all or some of the "molecules" of the ferments become "shocked," paralyzed, inactivated, and that if in that stage the shaking is not continued, these shocked molecules may recover and become active again. We

can say that in our experiments not a single fact was observed which could have been interpreted in that way; but we are willing to admit the possibility of such an occurrence. But a few occurrences like that would not yet signify that the entire effect of shaking is mere inactivation. We may mention here that in the experiments by Meltzer with one of the water bacteria of which usually a complete destruction by shaking was obtained, it occurred sometimes that after keeping the cultures (unopened) for some time, some colonies would appear which at first would have a growth different from that peculiar to that organism, but gradually it would assume the character of the colonies of the original micrococcus. Such colonies were always only few in number. The interpretation of this phenomenon was that sometimes a few of the organisms survived the fatal effect of shaking, but even these survivors received a shock, from which they recovered only slowly.

We have to call attention to another point. While shaking causes an effect which is specifically due to that factor, by the methods of shaking as they are employed at present in the still primitive stage of development of our subject, some of the injurious effects met with in some of the shaking experiments might be due to some other injurious factors which it is difficult to separate from the shaking effects. The Schmidt-Nielsens indicate such possibilities. It is possible, for instance, that the results of Harlow and Stiles may be due to two causes: to shaking and to adsorption.

#### DISCUSSION.

We are now coming to a discussion of the nature of the process which causes the destruction or inactivation of the ferment by shaking. We have stated in a previous section the hypothesis which led us up to this investigation. The results as far as they went are in agreement with the anticipation raised by that hypothesis. This, however, does not yet prove that our hypothesis is indeed the only explanation of this process. There are other possible interpretations of the phenomenon, and we shall now try to see the merits of them. We shall discuss first such interpretations as would explain the results of shaking by other than mechanical effects. That it is not due to an alkalinity produced by a solution of the glass was shown in our experiments by the fact

that the shaken solutions were still acid; that the effect was the same when the bottle was paraffined; and in the Schmidt-Nielsens experiments it was shown by the fact that the results were the same, even when the shaking was carried out in stone bottles. That it is not due to a rise of temperature caused by the shaking, as some are inclined to suppose, is proven by the fact that the direct test demonstrated that the rise did not reach even  $1^{\circ}$  C., and furthermore a rise of even  $10^{\circ}$  C. would never produce such a destruction in such a short time. That the destruction was not due to an oxidation by the oxygen of the air within the bottle was proven by the fact that the destructive effect remained the same when the space above the liquid within the bottle was filled with hydrogen or carbon dioxide.

Turning now to the physical explanations of the phenomenon, we have to mention that, according to the opinion of Abderhalden and Guggenheim, it is very probable that the precipitations which form by shaking pull down the ferments. But then they add that "since the loss of ferment activity occurs also in clear solutions, it is evident that a direct precipitation is not necessary for the inhibition of the ferment activity." Which, then, in their opinion, is, under these last-mentioned circumstances, the real cause of the inhibition of the ferment activity, the authors do not make clear. Harlow and Stiles say that "while we are convinced that the removal of the enzyme by contact with surfaces has been the chief factor in our experiments, we have seen some reason to believe in a secondary influence of the shaking, either an agglomeration or a disintegration of molecules it may be." The authors say that the effect of contact with surfaces "is analogous to the removal of enzymes from solutions by precipitates and filtration." The authors have probably in mind the phenomenon of adsorption of ferments. The Schmidt-Nielsens consider the inactivation by shaking as a new phenomenon and do not discuss the probable nature of it.

As far as we can see two explanations are open to those who are disinclined to assume that we deal here with a new phenomenon. One is that the ferments are carried down by a precipitate, and the second is that the ferments are removed from the solution by the adsorption to the wall of the bottle in which it is shaken. As to the first explanation, it cannot mean that it is carried down by visible precipitations, since the activity of the ferment is in-

hibited by shaking even when, as Abderhalden and Guggenheim state, the fluid remains perfectly clear. However, this view might find a support in Ramsden's observations<sup>24</sup> that shaking of a solution of albumen produces a coagulation, or, as Mann<sup>25</sup> says, a conglutination. It might then be assumed that the proteids closely connected with the ferments themselves coagulate and thus imprison the ferment; these fine particles, however, are perhaps too small to cause perceptible turbidity. But is it probable that these particles will be capable of holding the ferments imprisoned forever? Moreover, why should this infinitesimal amount of coagulated proteid not become rapidly digested by the pepsin and the HCl which are present in abundance in these solutions? Furthermore, according to Ramsden, the mechanical proteid coagula are dissolved in alkaline solutions becoming alkali-albuminates. How should, then, the trypsin ferment in the alkaline solution be imprisoned in these proteid masses, and why should the latter not be digested? It seems to us that this interpretation is far from being plausible.

The second interpretation would assume that by shaking a solution of ferment in a glass bottle the entire quantity of the ferment would be withdrawn from the solution and become adherent to the wall of the smooth glass bottle, remaining there adherent permanently, and thus destroy the activity of the ferment. The basis for this assumption is, as indicated before, the well-known phenomenon, discovered by Von Wittich, of the adsorption of ferment to fibrin and to some other substances. We need not enter into a discussion of the probability of such an assumption. It suffices to call attention to the fact that among the experiments on adsorption of pepsin we find such ones in which after shaking pepsin solutions for an hour in a glass bottle, even with an admixture of a quantity of powdered glass, no adsorption took place, either to the wall of the bottle or to the powdered glass; the entire quantity of pepsin was recovered from the filtrate.<sup>26</sup> It seems to us that, on the contrary, some phenomena which were considered as being due to adsorption might have to be studied over again, since in many of the cases the phenomena were obtained after

<sup>24</sup> RAMSDEN: *Archiv für Physiologie*, 1894, p. 517.

<sup>25</sup> MANN: *Chemistry of proteids*, 1906, p. 273.

<sup>26</sup> DAUWE: *HOFMEISTER's Beiträge zur chemischen Physiologie*, 1905, vi, p. 426.

shaking the solutions for an hour or longer and the question could be raised how much of the phenomenon was due to shaking.

At all events, it seems to us that the various attempts to explain the destructive effect of shaking upon ferments by *known* chemical or physical processes are far from plausible and have, so it seems to us, a less satisfactory basis than the hypothesis upon which, at least in our experiments, the facts of the destruction of the proteolytic ferments by shaking were discovered. We now turn to a discussion of this hypothesis.

**Our theory.** — In the first place, it seems to us that the destruction of the ferments by shaking is analogous to the destruction by shaking of organized, living bodies of microscopical dimensions. For bacteria, yeasts, red blood corpuscles, and echinoderm eggs the profound influence of shaking is now, as it was shown above, a well-established phenomenon. From this point of view the fact of the destruction of ferments by shaking is therefore an entirely new phenomenon no longer. We go further and assume that the nature of the process of destruction by shaking is in both instances the same. Ferments, of course, differ greatly from living organisms. But even red blood corpuscles differ considerably from living organisms, — they are incapable of reproduction and probably also of growth. Nevertheless we do not hesitate to designate them as cells, and certainly as living cells. Our assumption is that ferments have a certain structure, an organization; that this organization they have in common with living organisms and red blood cells, and that shaking affects all three categories of beings by attacking this structure. Life is something in addition; but that structure is indispensable to life. On the other hand, it is this structure which distinguishes organized bodies from simple aggregates of colloid organic matter. These two kinds of bodies react in a fundamentally different way to heat, light, or shaking. We shall discuss here only the difference in the reaction to shaking. Shaking breaks down organized bodies by molecular disintegration, by converting them to dust; shaking affects colloid organic tiny clumps by uniting, coalescing them to masses somewhat larger in size than before, just as was observed by Ramsden that shaking of solutions of proteid brought about threadlike formations. This contrast in the effect of shaking was observed by Meltzer in continuous shaking of red blood corpuscles. The first effect of the shaking was the gradual conversion of the corpuscles into dust;

the continuous shaking of this dust, however, converted it into threadlike ragged masses.

We distinguish, then, between living bodies, organized bodies, and unorganized colloid, organic masses. *Living bodies are organized bodies plus life*, and shaking attacks the organization in both kinds of organized bodies in the same manner. Ferments are organized bodies (not to confound with the older expression of organized ferments), and shaking attacks their structure in the same manner as it attacks living cells. In both cases the destruction is in the nature of a molecular disintegration.

This is one part of our theory or rather one of the hypotheses. There is another part to our theory, another hypothesis which is however not indispensable to the support of the first one. It refers to the nature of the organization of the structure of these bodies. It assumes that in organized bodies the physical molecules are united into groups, physiological units, which are disconnected among themselves and are surrounded by layers of fluid which are connected throughout the entire organized body. This organization permits the vibration of the physiological units, which is transmitted to them from outside (from the continuous vibrations going on in the inorganic world or from the shocks within the living complex organism), and which is essential for carrying on metabolic processes throughout all or many of the organized bodies.<sup>27</sup> Violent shaking causes a sudden disintegration of this organization, a *disbanding of the physiological units*. (Temperature affects the very same structure, hence the greater effect of shaking under higher temperatures.) It is self-evident that this organization will vary greatly in its details in the different species as well as among the individuals of the same species of organized bodies. Shaking, therefore, will affect differently different species, and also differently some individuals of the same species. Hence the rapid inactivation of the greatest part and the resistance of the remaining part of shaken ferments, and hence the difference in the resistance to shaking between different ferments or between different living cells.

In unorganized colloid organic matter the relations are perhaps just the reverse: a continuous connection between irregular groups

<sup>27</sup> We do not wish to express here an opinion with regard to the occurrence of metabolic processes of some kind in ferments; our theory does not require such an assumption.

of the fine solid particles by means of threads, fibres, pellicles, and a disconnection between the enclosed liquids. Shaking, therefore, in a general and violent way causes a coalescence of the solids and not a disintegration.

The details of the organization and the character of the vibrations of the physiological units in ferments probably differ greatly from that in other organized bodies and differ specifically in each enzyme.

#### NON-SPECIFIC AND SPECIFIC VIBRATION.

In the foregoing we stated that the vibrations of the physiological units, within the living cells as well as within ferments, have their origin in external causes, in external shocks, shakings, and vibrations of all sorts. These external non-differentiated shocks communicated simultaneously to several organized bodies may produce in each body vibrations peculiar to that body on account of the specificity of the organization. There may be, however, such external shakings which are better adapted to the vibrations of one body than to those of another; they are in this case adequate or specific shakings. We may then go a step further and say that the vibrations of these organized bodies may again in their turn affect other bodies in a general as well as in a specific way. In mixtures of bacteria the lively moving organisms may affect their neighbors by favoring the metabolism, accelerating the process of division and hastening the breaking down of the decrepit individuals (Meltzer<sup>28</sup>). This is an instance of a general effect. As a specific effect we may perhaps cite the action of the motile spermatozoön. It is possible that *the shock which the ovum receives from the impact of the speeding spermatozoön is an accelerating mechanical factor in the cleavage processes which follow within the ovum*. These shocks are perhaps indeed specific ones: the specific movements of the starfish sperm are adequate for the starfish egg, and the specific motility of the arbacia sperm is adequate for an arbacia egg (Meltzer<sup>29</sup>). Whereas artificial shakings are effective for both eggs only when violent and when causing destruction; mild non-specific shaking starts cleavage only in starfish and amphitrite eggs, it does not affect arbacia eggs.

<sup>28</sup> MELTZER: Zeitschrift für Biologie, 1894, xxx, p. 464.

<sup>29</sup> MELTZER: This journal, 1903, ix, p. 245.

## *Effect of Shaking upon the Proteolytic Ferments. III*

Extending this very assumption to the ferments, we may perhaps state that the vibrations of ferments exert in some instances a non-specific effect, causing a variety of cleavages in various substances, and in other substances the action is definitely specific — like a key to a lock. This leads up to the revival of Naegeli's theory that ferment action is due to a specific molecular vibration, — into a discussion of which, however, we shall not enter, as it is outside of the scope of the present paper.

We may, however, point out very briefly that the so-called inorganic ferments have a structure similar to the one we assumed to be possessed by the enzymes, *i. e.* extremely fine, discrete, solid particles surrounded by very fine connected films of fluid.

We have assumed that living cells and enzymes are built up of physiological units. We wish to say that these units have nothing to do with Verworn's Biogenes, Adami's Biophores, or Rubner's Bionts. Or, more correctly, we wish to express no opinion as to the relations in which our physiological units may stand to the units of life. We do not discuss the nature of life in this paper. We only assume that bodies which are affected by physical factors like vibration, heat, and light, in a similar manner have a similar organization upon which the physical factors exert their influence. Life is something in addition to this organization. What life is, what its units may be, are questions with which we do not deal in this paper.

### SUMMARY.

The more essential results of our experiments are the facts that shaking may completely destroy the three ferments, — pepsin, renin, and trypsin; that they are destroyed more rapidly at higher than at lower temperatures; that trypsin is more easily destroyed than pepsin; and that the shaking produced by the respiratory movements is capable of causing some destruction of the ferments.

Recent experiments by other investigators show that also other ferments may be inactivated by shaking.

Numerous older experiments have established that shaking is capable of influencing fundamentally bacteria, yeast, red blood corpuscles, and echinoderm eggs.

The assumption is here made that the nature of the destruction

of ferments is similar to that which takes place in the destruction of living cells, and that shaking affects a certain structure which is common to living cells as well as to red blood corpuscles and to ferments. The further details of this theory cannot be included in the summary.

## THE EFFECT OF SUBMINIMAL STIMULATION OF THE PNEUMOGASTRIC NERVES UPON THE ONSET OF CARDIAC RIGOR.

By DON R. JOSEPH AND S. J. MELTZER.

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FOR skeletal muscles it is well established that section of the motor nerves retards, and stimulation of the peripheral ends of these nerves hastens, the onset of rigor mortis in the corresponding muscles. On the hypothesis that stimulation of inhibitory nerves may cause a retardation of the onset of rigor, we carried out a series of experiments upon cardiac rigor in which the pneumogastric nerves were stimulated. We published recently a full account of that investigation.<sup>1</sup> The chief result was that the onset of rigor, instead of being retarded as expected, was definitely accelerated. In those experiments the vagi were treated with *effective* stimuli, that is, there were considerable slowing and stoppages of the heart beats. Our interpretation of the result was that the acceleration of the phenomena of rigor was caused by premature cardiac asphyxia, brought on by the great slowing and standstills of the heart. Notwithstanding this result, stimulation of the inhibitory nerve fibres might well have a tendency to retard the onset of rigor, but the retardation in those experiments may have been overcompensated by a hastening due to local asphyxia of the cardiac muscles. This consideration gave rise to the problem which is dealt with in the present paper, — the question whether *ineffective* stimulation of the vagi would bring out the anticipated retardation of the rigor.

The fact that rigor of skeletal muscles appears earlier with nerves intact than with nerves cut was interpreted to mean that subminimal stimuli are continually transmitted from the central nervous system

<sup>1</sup> JOSEPH and MELTZER: Journal of experimental medicine, 1909, xi, pp. 10 and 314.

to the muscles, causing thereby a hastening of the postmortem rigor. In Hermann's laboratories, where the question of the relations of the nervous system to the onset of rigor received manifold attention, Bierfreund<sup>2</sup> approached the subject experimentally and obtained the surprising result that the leg, the cut sciatic of which was stimulated, developed rigor later than the control leg. However, Gotschlich<sup>3</sup> found that subminimal stimulation brought on acidity of the muscle, and B. Danilewsky<sup>4</sup> observed the production of heat in the muscle by subminimal stimulation. These facts caused Meirowsky<sup>5</sup> to take up again (in Hermann's laboratory) the question of the influence of subminimal stimulation upon rigor. The result was this time that treating motor nerves with subminimal stimuli hastens the onset of rigor. Of fourteen experiments twelve were positive, one gave no result, and one had an opposite result, that is, the onset of the rigor was retarded. On the basis of this result we again undertook the study of cardiac rigor under the influence of stimulation of the vagi, using, however, this time *ineffective stimuli*, that is, stimuli which were not capable of influencing the heart beats in a perceptible manner.

**Methods.** — In the previous paper we have shown that in the researches upon cardiac rigor by various investigators, in which the graphic method was employed, a serious error was introduced in the results. The filling up of the ventricular cavity with some fluid for the purpose of connecting it with a manometer caused a tonic contraction of the heart which at some time later went over into rigor without the recognizable occurrence of an intermediate relaxation. Some of the investigators assumed therefore that rigor begins immediately after death. In our observations we left the heart *in situ*, handling it as little as possible. The onset and progress of rigor were judged simply by inspection and palpation, which give reliable results indeed, especially after a little practice. Regarding the particulars of this method we must refer to our previous paper. We wish to mention, however, that in our previous observations we established the fact that three definite periods are to be distinguished in the conditions of the heart between death and maximum rigor. In the first period the ventricles

<sup>2</sup> M. BIERFREUND: *Archiv für die gesammte Physiologie*, 1888, xliii, p. 203.

<sup>3</sup> GOTSCHLICH: *Ibid.*, 1894, lvi, p. 363.

<sup>4</sup> B. DANILEWSKY: *Ibid.*, 1889, vi, p. 353.

<sup>5</sup> MEIROWSKY: *Ibid.*, 1899, lxxviii, p. 64.

show more or less definite although inefficient spontaneous contractions. In the second period the heart is neither beating nor is it in rigor; it is then more relaxed than during a normal diastole. The third period comprises the time which elapses from the beginning of rigor to the attainment of its maximum.

During the second period the ventricles gradually lose their irritability. All periods are much longer in the right than in the left ventricle. The irritability also disappears later in the right than in the left ventricle.

In this series as well as in the former already published the death of the animals was brought about by exsanguination. There was, however, this difference in the method between the two series of investigations: while in the previous experiments the exsanguination was brought on by opening both carotid arteries, it was accomplished in this series, at least in the main experiments, by opening the abdominal aorta. Exsanguination by this method is more complete than by bleeding from the carotid arteries. The respiration usually stopped about five minutes after beginning the bleeding. The thorax was then opened, the heart freely exposed, and the ascending aorta and pulmonary artery opened near their origin. Through these openings the remaining intraventricular blood or clots were gently removed.

For each experiment two animals were used, both as nearly of the same size as possible. Both were etherized and tracheotomized. The etherization was continued in both animals for about one hour. This was done to have both animals under exactly the same conditions, especially since it was found in the last investigations that ether retards the onset of cardiac rigor. In the experiments which gave us our main result, both vagi were exposed and cut in both animals. In one of these animals both vagi were stimulated continually with induction currents for one hour. At the beginning of each experiment the strength of current (the distance of coils) was ascertained, which gave a minimum effect on the heart; then the secondary coil was moved back 100 or 150 mm. With this strength of current, which exerted no perceptible effect, both vagi were stimulated for about one hour, at the end of which both animals were killed in the manner described above.

**Results.**—From ten pairs of dogs—A, control, and B, upon which the experiments were carried out in the manner just described—we obtained the following time averages for the dura-

tion of the various periods or states intervening between death and maximum rigor of the heart. The average time which passed between death and the beginning of rigor (which for our purpose is the most important period) was for the left ventricle in dogs A (controls) seventy-five and in dogs B one hundred and seven minutes; for the right ventricle in dogs A one hundred and nine and in dogs B one hundred and forty-three minutes. In other words, the onset of rigor was retarded in the animals in which the vagi were stimulated — for the left ventricle by thirty-two minutes and for the right ventricle by thirty-four minutes.

The average time which passed between death and stoppage of all spontaneous contractions was for the left ventricle in dogs A twenty-one, and in dogs B forty-seven minutes; for the right ventricle in A twenty-two and in B forty-nine minutes. This means again that in the dogs in which the vagi were stimulated the left ventricle continued beating after death and complete exsanguination twenty-six minutes, and the right ventricle twenty-seven minutes longer than in the control.

The average time which passed from the beginning of rigor until it reached its maximum was for the left ventricle in dogs A eighty-two, and in dogs B eighty-six minutes; for the right ventricle in A seventy-two, and in B seventy minutes. In other words, the average time for the development of rigor in both ventricles from its beginning until it reached the maximum was practically the same for both animals.

The average time which passed between complete stoppage of all contractions and the beginning of rigor — the relaxation period — was, for the left ventricle in dogs A fifty-four, and in dogs B sixty minutes; for the right ventricle in A eighty-eight, and in B eighty-three minutes. In other words, the relaxation time was slightly longer for the left ventricle in the stimulated animal and slightly longer for the right ventricle in the control animal — which means there was but little difference in either direction.

After the ventricles stopped all spontaneous contractions, they were tested either by mechanical stimulation or by electric shocks, as to their irritability. The average time which passed from the stoppage of spontaneous contractions until all irritability ceased, was for the left ventricle in dogs A forty-four, and in B fifty minutes; for the right ventricle in dogs A sixty-eight, and in B eighty-nine minutes. In other words the irritability of the heart,

after stoppage of pulsations in the dogs in which the vagi were stimulated, persisted in the left ventricle longer by six, and in the right ventricle longer by twenty-one minutes than in the hearts of the controls.

Including the irritability of the heart during the period of pulsation, we have the following figures: The average time from death to complete loss of irritability was for the left ventricle in dogs A sixty-five, and in dogs B ninety-seven minutes; for the right ventricle in dogs A ninety-eight, and in dogs B one hundred and thirty-eight minutes. That is—the irritability persisted after death thirty-two minutes longer in the left ventricle and forty minutes longer in the right ventricle of the experimental animals than in the controls.

Maximum rigor is a more definite landmark than its beginning. We shall therefore give here also the average time which passed between death and maximum rigor. For the left ventricle in dogs A it was one hundred and fifty-six, and in dogs B one hundred and ninety-three minutes; for the right ventricle in dogs A one hundred and eighty-two, and in B two hundred and twelve minutes. In other words, the average time for the interval between death and maximum rigor was prolonged in the dogs in which the vagi were stimulated, for the left ventricle thirty-seven, and for the right ventricle thirty minutes.

As to the making up of the averages given in the above data, it must be stated that for the periods which were prolonged in B (stimulated vagi) there was in each case one exception, and in one or two instances two exceptions, that is, cases in which either for the left or for the right ventricle the period for A was prolonged over that for B. However, this prolongation amounted in most cases to a few minutes only, and even this was doubtful in some. On the other hand, in the period of relaxation, as well as that of development of rigor, there were prolongations on one side as many times as on the other.

To recapitulate the results briefly: in nearly all the dogs in which both pneumogastric nerves were stimulated antemortem for one hour in an ineffective manner, that is, with electric stimuli which were incapable of producing a perceptible effect upon the heart beats, there was a definite effect upon the events in the heart after death. These effects were: the onset of rigor was retarded; the completely exsanguinated ventricles beat longer; and their

irritability persisted longer than that of the control animals. The slight shortening of the relaxation period in the stimulated animals might be only a secondary phenomenon and due to the prolongation of the preceding pulsation period. It is a known fact that contractions of a muscle hasten the oncoming of its rigor.

In connection with the foregoing results the following observation is of interest. On three pairs of dogs the following experiments were carried out. In each experiment both dogs were etherized and tracheotomized; then in one dog both vagi were cut, while in the other they were left intact. After a period of seventy-five minutes (or longer) both dogs were killed about the same time by bleeding from the abdominal aorta. The average time of all periods was prolonged in the animals the nerves of which were not cut, with the single exception of the period of pulsation in the right ventricle. Even the average time of the period of development of rigor was retarded in the animals with intact vagi. The cutting of the vagi, then, accelerated the course of the various postmortem phenomena. Since the cardiac vagi of the dog are normally in a state of tonus, we may say that the normal tonus of the vagi retards the onset and development of cardiac rigor. The effect of the tonus, of course, is distinctly only inhibitory in character. These results, therefore, support the assumption that inhibitory impulses retard the onset of rigor. The above three experiments showed further, as mentioned before, that the inhibitory impulses retard also the *development* of the rigor. This was not the case in our stimulation experiments. We must remember, however, that in these experiments we have employed subminimal stimuli, which, with regard to their effect upon the inhibitory nerve fibres, were probably too weak; the artificial inhibitory impulses which they sent to the ventricles were surely weaker than those which are sent normally through the intact vagi, since they produced no such slowing as does the tonus. Had we employed somewhat stronger stimulation, it may have occurred that the development of rigor would also have been retarded. Furthermore, comparing our experiments with those of Meirowsky on motor nerves, we find that the periods of our stimulation were a good deal shorter than the ones employed by this investigator, who stimulated two and one-half, five, and even twenty hours. Here again it is possible that we could have obtained still more striking effects had we extended the period of our stimulation.

SUMMARY.

Antemortem stimulation of the peripheral ends of the pneumogastric nerves with electric currents too weak to produce a perceptible effect upon the heartbeats, prolongs the spontaneous contractions and the irritability of the ventricles after death, and retards the onset of rigor.

It is probable that the relation of inhibitory nerves to cardiac rigor is the reverse of that of motor nerves to the rigor of skeletal muscles.



## THE PURINES AND PURINE METABOLISM OF THE HUMAN FETUS AND PLACENTA.<sup>1</sup>

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In the study of the development of the several enzymes involved in purine metabolism in the embryo pig, Mendel and Mitchell<sup>2</sup> found that these several enzymes do not appear together, but one at a time, and some not until late in antenatal or early in postnatal life. Even at the earliest stages studied, 50 mm. embryos, the enzymes which liberate the purines from the nucleoproteins are present, the *nuclease* of Ivanoff. The deamidizing enzymes are also present at a correspondingly early stage, *adenase* being demonstrated by the reduction of the quantity of adenine and increase in hypoxanthine in autolyzing livers from pigs of 50 mm., 75 mm., and 100 mm. length.<sup>3</sup> *Guanase* is absent from the liver of the pig at all times, in the adult as well as in the fetal animal, but it could be demonstrated in the other viscera of embryo pigs of 120 mm. length, the smallest examined for this enzyme, by the conversion of guanine into xanthine by extracts of the combined organs. The next step in purine metabolism is the conversion of hypoxanthine and xanthine into uric acid by a process of oxidation, which is accomplished by an oxidizing enzyme or enzymes, the *xantho-oxidase*

<sup>1</sup> This work has been aided by a grant from the Rockefeller Institute for Medical Research.

For materials we are indebted to several Chicago physicians, especially to Dr. J. W. Jobling of the Michael Reese Hospital. Much assistance in the analytical work has been given by Mr. J. H. Mitchell.

<sup>2</sup> *Amer. Journ. of Physiol.*, xx, p. 97, 1907.

<sup>3</sup> Jones and Austrian (*this Journal*, iii, p. 227, 1909) could find no evidence of adenase in the liver until pig embryos have reached a length of 150 to 170 mm.

of Burian. This enzyme could not be detected in the livers or other viscera of embryo pigs up to 230 mm., the largest studied, but was present in the liver of a sucking pig about seven weeks old; intermediate stages were not examined, so the exact time of appearance of this enzyme cannot be stated. As the last step in purine metabolism in the pig, the uric acid formed by the other enzymes is destroyed by yet another oxidizing enzyme, the *uricase* of Battelli and Stern.<sup>1</sup> This enzyme is also missing in the liver or other tissues of fetal pigs of 200 mm. or less, and seems to be but feebly developed in the liver of sucking pigs two months old, although very active in the liver of adult pigs. Mendel and Mitchell remark "that the tardy appearance of the oxidative and katabolic enzymes concerned in the transformation of the purines is suggestive as a characteristic of growing, synthetic organs."

This development of the enzymes of purine metabolism, step by step, furnishes an interesting demonstration that the biological law, that the individual in its development reflects the entire developmental history of the species to which it belongs, may hold just as true in biochemistry as in morphology. The purine enzymes of the pig at different stages of development reflect the conditions in different forms of life, for the simplest forms would seem, as far as the investigations yet made show us, to have a correspondingly simple outfit of purine enzymes. As the scale of complexity increases, the number of these enzymes increases until in the adult mammal we have the maximum.<sup>2</sup> Thus in the yeast there is nuclease, as shown by the appearance of free purines during autolysis of yeast, and also a single deamidizing enzyme, guanase, but no adenase or xantho-oxidase.<sup>3</sup> An invertebrate organism, the mollusk *Sycotypus canaliculatus*, has been shown to possess nuclease, guanase, and adenase, but no xantho-oxidase or uricase.<sup>4</sup> The embryo pig at 200 mm. or less is, therefore, equipped with a scanty number of purine enzymes, similar to the yeast or the mollusk. When later it

<sup>1</sup> *Compt. rend. soc. biol.*, Paris, lxvi, p. 612, 1909.

<sup>2</sup> Compare Herlitzka: *Arch. ital. de biol.*, xlviii, p. 119, 1907, "Sur l'ontogénèse des ferments."

<sup>3</sup> Straughn and Jones: *This Journal*, vi, p. 245, 1909.

<sup>4</sup> Mendel and Wells: *Amer. Journ. of Physiol.*, xxiv, p. 170, 1909.

acquires the xantho-oxidase, but is still lacking the uricase, it would seem to be in the same condition as the birds and reptiles, which form uric acid but seem to be entirely unable to destroy it. The uricolytic property is a later development, and in man this would seem never to be acquired, or at least not actively,<sup>1</sup> in which respect man is behind the cow, dog, rabbit and guinea-pig, all of which animals can destroy uric acid readily.<sup>2</sup>

In the following article it will be shown that the sequence of enzyme formation in the human fetus is quite similar to that observed by Mendel and Mitchell and by Jones and Austrian in the fetal pig.

#### ADENASE AND GUANASE.

The mature human fetus possesses both adenase and guanase, as shown by the following experiments:

*Experiment I.*—A full-term female fetus, weight 3400 grams, which died at birth because of lack of attendance. After removing the viscera for use in uricolysis experiments, the rest of the fetus, weighing 1835 grams, was ground up and allowed to autolyze in 5500 cc. water, after adding the solid residues of the viscera left after extracting them over night in water, and straining through muslin. (The extrats were used in uricolysis experiments.) Autolysis was continued for 38 days at 36–38° C., without access of air to the autolyzing mixture which was covered by a thick layer of toluol. The mixture was found to be distinctly acid to litmus in spite of the presence of the bone salts. The coagulable proteins were removed by coagulation and filtration, and the purines were precipitated by the copper sulphate and sodium bisulphite method of Krüger and Solomon. The purines were freed with hydrogen sulphide and fractionated in the usual way. There was no purine present that was not readily soluble in 1 per cent ammonia solution, showing the absence of *guanine*, but there was a large amount of purine coming out in crusts and scales which was insoluble in a considerable volume of water at 35° C. After being purified this material gave the characteristic reaction of *xanthine*, and was entirely free from uric acid. The amount of xanthine recovered was 0.7 gram.

<sup>1</sup> See Wells and Corper: This *Journal*, vi, p. 321, 1909.

<sup>2</sup> According to Jones and de Angulo (*Proc. Amer. Soc. Biol. Chemists*, i, p. 193, 1909) the entire equipment of purine enzymes in the dog's liver is acquired only after birth, not even guanase or adenase being present in newly born dog's liver.

The filtrate from the xanthine did not give any precipitate with picric acid, showing the absence of adenine. It gave, however, an abundant precipitate with ammoniacal silver chloride, which on being redissolved in hot dilute nitric acid gave a large crop of typical crystals of hypoxanthine silver nitrate, weighing 0.712 gram after drying at 120°C. This corresponds to 0.266 gram of *hypoxanthine*.

The presence of such an abundance of free xanthine and hypoxanthine in the autolyzing mixture and the absence of guanine and adenine indicates the presence of both *guanase* and *adenase*. It would also seem that guanine is more abundant than adenine in the fetal tissues, in view of the relative excess of xanthine over hypoxanthine.

Studies of earlier stages show an interesting condition, namely, that the adenase and guanase do not appear at the same time, the guanase being found earlier than the adenase. This is shown by the two following experiments with fetuses of almost identical age:

*Experiment II.*—A fetus of about the third month, 8 cm. long, weight 9 grams, was ground up, and to it was added in solution 0.039 gram adenine hydrochloride (0.029 gram adenine) and 0.045 gram guanine hydrochloride (0.034 gram guanine). Autolysis was continued 37 days at 37°C., without air. At the end of that time, when analyzed, the guanine had entirely disappeared, being replaced by *xanthine* of which 0.014 gram was recovered. On adding picric acid to the filtrate from the guanine a voluminous precipitate of typical adenine picrate was obtained, which had a melting point of 273° (uncorr.), and weighed 0.057 gram, corresponding to 0.021 gram *adenine*. After removal of the adenine picrate the filtrate was found to still give a small precipitate with copper sulphate and sodium bisulphite. On decomposing with hydrogen sulphide and reprecipitating with ammoniacal silver chloride a slight flocculent precipitate was obtained indicating the presence of a trace of some purine, but the amount was too small to identify; presumably it was either a trace of hypoxanthine derived from the fetal tissues or traces of one of the other purines which had failed to be removed in the earlier steps of the analysis.

This experiment indicates that in the fetus at three months guanase is present, but adenase is still absent. It is well corroborated by a duplicate experiment with a fetus of almost exactly the same size.

*Experiment III.*—A three-months fetus, obtained by operation from a freshly ruptured tubal pregnancy. Length 6.5 cm., weight 8 grams. Ground up and placed in a solution containing 0.0962 gram guanine hydrochloride (0.074 gram guanine) and 0.1041 gram adenine hydrochloride

(0.078 gram adenine). Let autolyze without air for 13 days at 37°. Recovered 0.046 gram *xanthine*, no guanine, and 0.1501 gram adenine picrate (corresponding to 0.056 gram *adenine*) with a melting point of 270° (uncorr.). After removal of the adenine picrate there was still a minute amount of purine precipitable by copper sulphate, which was freed from copper with hydrogen sulphide, reprecipitated with ammoniacal silver chloride, and recrystallized after solution in boiling, dilute nitric acid. A few milligrams (0.029 gram) of hypoxanthine silver nitrate crystals were obtained, corresponding to about 10 mg. of *hypoxanthine*.

These two experiments furnished convincing evidence of the independence of the two deamidizing enzymes, adenase and guanase, which was disputed by Schittenhelm but may be now considered to be conclusively settled by the results obtained by Jones and Mendel and their co-workers.<sup>1</sup>

Between the third and fifth months the adenase makes its appearance, as shown by the next experiments.

*Experiment IV.*—Fetus about fifth month, 26 cm. long, weight 450 grams, weight of liver 27 grams. Made separate emulsions of the ground-up liver and the remainder of the fetus, and to each added guanine hydrochloride and adenine hydrochloride. Autolysis was continued at 37°, without air, for 50 days, and then the free purines were isolated. It was found that the adenine and the guanine had both entirely disappeared, their place being taken by xanthine and hypoxanthine, the results of quantitative determinations being as follows:

(A) Liver, weight 27 grams. Added 0.1080 gram adenine hydrochloride (0.080 gram adenine) and 0.080 gram guanine hydrochloride (0.061 gram guanine). Recovered 0.0596 grams *xanthine* and 0.0688 gram *hypoxanthine* (0.1841 gram hypoxanthine silver nitrate).

(B) Fetus, weight 420 grams. Added 0.1105 gram adenine hydrochloride (0.0825 gram adenine) and 0.1230 gram guanine hydrochloride (0.0947 gram guanine). Recovered 0.1524 gram *xanthine* and 0.118 gram *hypoxanthine* (0.3165 gram hypoxanthine silver nitrate).

<sup>1</sup> It may be mentioned that Schittenhelm himself has obtained excellent evidence of the existence of two separate deamidizing enzymes acting respectively on adenine and guanine, for he reports (Schittenhelm and Schmidt, *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 428, 1907) that the lung of the human fetus has the power of converting guanine into xanthine, although it does not convert adenine into hypoxanthine (Expts. 15 and 16). Apparently he failed to appreciate the significance of this result, since a few pages later (pp. 432-437) he tries to show that Jones is in error in maintaining the existence of two separate enzymes.

These results indicate a conversion of the adenine and guanine added to the liver to hypoxanthine and xanthine which is practically quantitative; in the case of the entire fetus there has been some gain in the xanthine and hypoxanthine, presumably from autolysis of the 420 grams of tissue that were present in the mixture.

*Experiment V.*—Male fetus, about the fifth month, length 25 cm., weight 235 grams. This was ground up, put in one liter of water and toluol to which had been added 0.0986 gram adenine hydrochloride (equivalent to 0.072 gram adenine) and autolysis continued without air for 12 days at 37°. The free purines were precipitated and isolated by the usual method. No guanine was found, but a trace of what seemed to be xanthine. Little or no adenine remained, since but a faint turbidity was produced with picric acid, and that only after standing for some time. The chief purine obtained was *hypoxanthine*, which, isolated as the hypoxanthine silver nitrate, weighed 0.1752 gram, corresponding to 0.0654 gram hypoxanthine. Evidently, therefore, the adenine has been converted into hypoxanthine.

*Experiment VI.*—A female fetus, weight 605 grams, length 32 cm., probably about the sixth month. The entire tissue was ground up and permitted to autolyze for 43 days under toluol, without air, in 2500 cc. of water, about half the time at 37° and the rest of the time at room temperature. After removal of the insoluble and coagulable materials the purines were precipitated and isolated in the usual way. No guanine, uric acid or adenine was recovered, but there was obtained 0.283 gram *xanthine* and 0.412 gram hypoxanthine silver nitrate, corresponding to 0.154 gram *hypoxanthine*.

These last three experiments establish the presence of both *adenase* and *guanase* in the liver and other tissues of the human fetus at the fifth and sixth months. Experiment IV also proves that the human liver possesses both these enzymes, in contrast to the liver of the pig which has only *adenase*, and the liver of the dog which has only *guanase*.<sup>1</sup>

#### XANTHO-OXIDASE.

The following experiments show that, as in the pig, the oxidizing enzyme appears later than the deamidizing enzymes, but yet some time before birth of the human fetus.

<sup>1</sup> Jones and de Angulo: *loc. cit.*

*Experiment VII.*—Fetus, said to be 8½ months, but the centers of ossification at the end of the femur were about 4 mm. in diameter. Length 48 cm., weight 2745 grams. Died at birth from intra-abdominal hemorrhage. The various viscera were ground up separately, extracted over night at room temperature, strained, and after addition of 0.1 gram xanthine to each, permitted to autolyze for 48 hours at 37° with a current of air bubbling slowly through all the time. Analysis of the mixture after this time gave the following results:

Tissue.	Material added.		Material recovered.	
1. Liver (168 gm.).....	0.1 gm. xanthine	.....	0.1565 gm. uric acid.	
2. Muscle (109 gm.).....	0.1 " "	.....	0.0952 " xanthine.	
3. Intestine (41 gm.).....	0.1 " "	.....	0.0963 " "	
4. Kidneys (20 gm.).....	0.1 " "	.....	0.1013 " "	
5. Spleen and Thymus (13 gm.).....	0.1 " "	.....	0.0914 " "	

From this experiment it would seem that in a fetus just before birth there is present in the liver an oxidizing enzyme which produces uric acid not only from the xanthine added to it, but also apparently from the purines of the liver itself. The other viscera and the muscle seem to be devoid of xantho-oxidase.

*Experiment VIII.*—A full-term infant, which lived but three hours on account of a congenital heart defect. The liver (weight 200 grams), the kidneys (weight 22 grams), and the remaining viscera (total weight 190 grams), were ground, extracted over night, and strained. The liver and viscera extracts were divided into equal parts, and after adding uric acid or xanthine as indicated below, the five extracts were allowed to autolyze at 37° for 48 hours with a constant current of air bubbling through. The results of analysis of the resulting mixtures were as follows:

Tissue	Substance added		Recovered		Per cent recovered
1. Liver (100 gm.)....	0.1514 gm. uric acid.	....	0.2002 gm. uric acid		132
2. Liver (100 gm.)....	0.0958 " xanthine.	....	0.0856 " "		
3. Viscera (95 gm.)...	0.1514 " uric acid.	....	0.1706 " "		112
4. Viscera (95 gm.)....	0.0958 " xanthine.	...	0.0463 " "		
			0.0687 " xanthine		
5. Kidney (22 gm.)...	0.1514 " uric acid.	....	0.1488 " uric acid		98

This experiment demonstrates that the liver and at least part of the viscera of the full-term fetus contain xantho-oxidase. The reason for the incompleteness of the conversion of the xanthine into uric acid by the mixed viscera may be the power of one

tissue to destroy the oxidizing enzymes of another, as observed by Künzel and Schittenhelm.<sup>1</sup> The absence of uricolytic enzymes is also shown by this experiment.

*Experiment IX.*—A well-developed, full-term normal male fetus, weight 3000 grams, which had been kept in an ice chest 36 hours, was ground up and let autolyze at 36° with air current, in 7000 cc. water and toluol. Autolysis was continued 23 days, the air current being run through for a day at a time at intervals. The free purines were separated as usual. No guanine or uric acid or adenine found, but 1.05 gram was of *xanthine* and 0.91 gram hypoxanthine silver nitrate, or 0.34 gram *hypoxanthine*.

Presumably the failure to demonstrate xantho-oxidase in this experiment depends upon the fact that this enzyme is very labile, and by the time the purines had been split out of the tissues, already dead 36 hours before autolysis began, the xantho-oxidase had been rendered inert. Also, as mentioned previously, the xantho-oxidase of one organ may be destroyed by the enzymes of another organ; hence experiments in which all the tissues are combined, as in this and the two following experiments, are of doubtful value.

*Experiment X.*—A fetus of about the third or fourth month, measuring 11 cm. and weighing 32 grams, was ground up, extracted with toluol-water over night at room temperature, and the extract strained through muslin. To it was added 0.0765 gram xanthine in solution, and it was permitted to autolyze under toluol with a constant stream of air bubbling slowly through for 48 hours. The free purines were then isolated in the usual manner, and found to consist chiefly of *xanthine*, of which 0.046 gram was recovered. No uric acid could be recovered, or even detected by the murexide test in the combined purines.

*Experiment XI.*—A fetus apparently between the fourth and fifth month, measuring 23 cm. long, and weighing 306 grams, was used for testing the presence of uricolytic enzymes and xantho-oxidase. It was ground up, autolyzed over night at room temperature, strained, and the extract divided into equal parts. Added 0.1445 gram uric acid to one (A) and to the other (B) 0.1405 gram xanthine. Let autolyze with air current for 48 hours, and recovered from A 0.1443 gram uric acid and from B 0.1407 gram xanthine. Therefore at this stage of development there seems to be neither xantho-oxidase nor uricolytic enzymes.

<sup>1</sup> *Zeitschr. f. exp. Pathol.*, p. 393, 1908.

In the next experiment the difficulty caused by the presence of several viscera in one mixture was avoided, at least as far as the liver is concerned, but the results were still negative.

*Experiment XII.*—Two fetuses, each about the sixth or seventh month, were obtained in a frozen condition, having been dead and frozen about 48 hours.

(A) weighed 1170 grams, length 37 cm., liver weighed 50 grams.

(B) weighed 755 grams, length 33 cm., liver weighed 40 grams.

The two livers were ground, united, extracted over night with toluol-water, strained through muslin, and to the extract 0.1012 gram xanthine was added. This mixture was autolyzed at 37° with a current of air for three days, at the end of which time 0.1077 gram xanthine was recovered, indicating the absence of active xantho-oxidase, and apparently a slight formation of xanthine from the purines of the liver extract.

United the remaining tissues of the two fetuses, weighing together 1835 grams, and let autolyze 26 days, with an air current passed through the mixture at intervals. Upon analysis of the free purines present at the end of autolysis, guanine, uric acid and adenine could not be demonstrated, but 0.52 gram xanthine and 0.65 gram of hypoxanthine silver nitrate (equal to 0.243 gram hypoxanthine) were recovered.

From these experiments it would seem that xantho-oxidase is present in the liver, possibly in other viscera, but not in the muscle, intestines, kidneys, spleen and thymus, at or shortly before birth. This enzyme seems to make its appearance after the sixth month of intra-uterine life, and before full term. Evidence is also obtained of the lability of the xantho-oxidase, as already demonstrated by Künzel and Schittenhelm, whereby it is readily destroyed during autolysis, and also by the presence of more than one organ in the extracts used for experiments.

#### PURINES AND PURINE METABOLISM OF THE PLACENTA.

As the fetus is so imperfectly equipped with purine enzymes in the earlier stages of its development, and has at no time the power of destroying the uric acid that its xantho-oxidase may form before birth,<sup>1</sup> it is of interest to consider what supplementary activities the placenta may have to help out the metabolism

<sup>1</sup> See the previous paper by Wells and Corper, this *Journal*, vi, p. 321, 1909.

of the fetus. The old custom of looking upon the placenta as merely a membrane through which substances were passively diffused, has been superseded by a recognition of the important metabolic activity of the placenta, which properly takes rank as an important, actively functioning organ. This view is emphasized in a recent article by Bergell and Falk,<sup>1</sup> in which it is shown that the placenta contains an intracellular enzyme capable of splitting tyrosine out of protein. Other investigations have shown that the placenta also possesses an enzyme or enzymes capable of synthesizing and hydrolyzing glycogen (Hoffbauer), and enzymes which split glucosides and esters (Higuchi).<sup>2</sup>

As a first step an analysis of fresh placenta was made, with particular reference to the quantity and nature of the purines present in this tissue.

#### ANALYSIS OF PLACENTA.

Three fresh human placentas, weighing 1530 grams together, were ground fine and placed in 7.5 liters of 6 per cent sulphuric acid for two days, until analysis could be done, the material being well mixed with acid to prevent autolysis. The water and total nitrogen were determined in two samples, with the following results:

NO.	FRESH WEIGHT	DRY WEIGHT		NITROGEN	
	gm.	gm.	per cent	gm.	per cent of fresh weight
.....	0.6607	0.1277	19.33	0.0177	2.68
....	1.0344	0.2014	19.47	0.0283	2.74

Average dry weight = 19.4 per cent; Nitrogen = 2.71 per cent of moist weight or 13.9 per cent of dry weight.

The acid mixture was boiled 12 hours, filtered and the residue left from the hydrolysis was hydrolyzed again 8 hours in 5 per cent sulphuric acid, and filtered. Neutralized the filtrates separately, made faintly alkaline with sodium hydroxide and then faintly acid with acetic acid. Filtered off the precipitate thus obtained, hydrolyzed this precipitate again separately and united the neutralized filtrate from this to the original solutions. Precipitated the purines from the filtrates separately by the copper sulphate method, obtaining but a small quantity from the second

<sup>1</sup> Bergell and Falk, *Munch. med. Wochenschr.*, lv, p. 2217, 1908.

<sup>2</sup> Higuchi, *Biochem. Zeitschr.*, xvii, p. 21, 1909.

hydrolysis solution, showing that the hydrolysis had been practically complete. United the purine precipitates, decomposed with hydrogen sulphide, filtered, washed thoroughly, and reprecipitated the purines with copper sulphate. After decomposing this second precipitate with hydrogen sulphide the filtrate was made up to 3500 cc. and two 35 cc. samples taken for nitrogen determination. These samples each contained 0.0087 gram N, equivalent to 0.87 gram purine nitrogen in the entire 1530 grams fresh placenta; therefore the purine nitrogen is 2.1 per cent of the total nitrogen. On the basis of about 45 per cent nitrogen in the purines the total amount of purines in this quantity of placenta is approximately 2 grams, corresponding to 0.13 per cent of the fresh weight, or 0.66 per cent of the dry weight. Burian and Schur<sup>1</sup> state that in 100 grams of fresh tissue the following amount of purine nitrogen is present; muscle, 0.06 gram; calves' thymus, 0.45 gram; calves' liver, 0.12 gram; spleen, 0.16 gram. In comparison with these results we find that in 100 grams fresh placenta there is 0.057 gram purine nitrogen, or 0.29 gram in each 100 grams dry tissue.

The purines were then isolated in the usual manner. No uric acid was found. An abundant residue, insoluble in ammonia was obtained, which weighed after repurification 0.62 gram, and which was identified as *guanine*.

A minute quantity of *xanthine*, approximately 0.030 gram, was obtained and identified.

Picric acid precipitated an abundant quantity of typical adenine picrate, weighing 1.5842 grams, which corresponds to 0.586 gram free *adenine*. Melting point 273° (uncorr.).

The purines of the filtrate from the adenine picrate were precipitated with copper sulphate, to free them from picric acid. After washing the copper salt of the purines free from picric acid it was decomposed with hydrogen sulphide, and the purines in the filtrate precipitated with ammoniacal silver chloride. This salt was dissolved in a minimum quantity of boiling dilute nitric acid, filtered hot, and from the filtrate there crystallized out on cooling 0.534 gram typical hypoxanthine silver nitrate, corresponding to 0.2 gram *hypoxanthine*.

To summarize, there were obtained from 1530 grams placenta 0.62 gram *guanine*, 0.03 gram *xanthine*, 0.586 gram *adenine*, and 0.2 gram *hypoxanthine*, a total of 1.436 grams of purines. This is somewhat below the 2 grams of purines that were to be expected from the quantity of nitrogen present in the purine precipitate, but the discrepancy is probably due largely to the unavoidable losses that occur in the process of isolation and purification of the individual purines. As the evidence obtained from other sources is to the effect that living tissues contain no free *xanthine*,

<sup>1</sup> *Arch. f. Physiol.*, lxxx, p. 308, 1900.

the presence of a minute quantity of this purine in the placenta may be explained in one of two ways: Either there was a slight degree of autolysis in the placentas during the few hours that elapsed after their expulsion and their immersion in the sulphuric acid, or else the xanthine may have been present as a result of autolysis in the necrotic, infarcted areas which are to be found in all full-term placentas.<sup>1</sup> The presence of so considerable a quantity of hypoxanthine, about one-seventh of the total purines, is in harmony with the general observation that hypoxanthine may occur free in tissues independent of deamidization processes, and may be taken as corroborating the views of Bergell and Falk<sup>2</sup> that the placenta is an organ where active metabolic processes are going on, and not a mere filter which simply regulates the quantity and quality of exchange between maternal and fetal blood.

#### PURINE ENZYMES OF THE PLACENTA.

In a preceding paper<sup>3</sup> it was shown that the human placenta at full term has no power of destroying uric acid, thus resembling all other human tissues studied. The results obtained by permitting extracts of placenta to act upon uric acid in the presence of a constant current of air are given in the following table:

Tissue.	Uric acid added.	Uric acid recovered.	Per cent recovered.
1. Placenta (boiled).....	0.1427 gm.....	0.1372 gm.....	96
2. Placenta (fresh).....	0.1513 " . . . . .	0.1467 " . . . . .	97
3. Placenta (fresh).....	0.1486 " . . . . .	0.1399 " . . . . .	94

The following experiments demonstrate the presence of adenase and guanase in placenta, and the absence of xantho-oxidase.

*Experiment XIII.*—980 grams of placental tissue, from two placentas, were ground up, placed in 2200 cc. water with toluol, and autolysis conducted at 37° with a current of air running through the mixture at intervals. After four days added 143 grams more of fresh placenta, to introduce fresh oxidizing enzymes, if such were present, to act upon the purines

<sup>1</sup> See Williams: *Johns Hopkins Hosp. Bull.* ix, p. 431, 1900.

<sup>2</sup> Bergell and Falk: *Münch. med. Wochenschr.*, lv, p. 2217, 1908.

<sup>3</sup> Wells and Corper: *This Journal*, vi, p. 321, 1909.

that might have been set free by this time. The autolysis was continued 30 days with intermittent flow of air, and in the filtrate from the coagulated protein was obtained 0.24 gram *xanthine* and 0.265 gram *hypoxanthine* (0.71 gram hypoxanthine silver nitrate).

As no free guanine or adenine was present we have evidence of the presence of both guanase and adenase, while the absence of uric acid indicates the probable absence of xantho-oxidase. It is interesting to observe that the amount of xanthine and hypoxanthine obtained was about equal, corresponding to the approximately equivalent amounts of guanine and adenine found by hydrolysis of the placenta, in contrast with the results obtained by autolysis of fetal tissues which always gave about twice as much xanthine as hypoxanthine.

*Experiment XIV.*—423 grams fresh placenta were ground, extracted over night at room temperature with 2 volumes of water and strained. To one-half the extract was added 0.1116 gram of xanthine and water to 500 cc. and the mixture allowed to autolyze at 37° with a slow current of air running through it constantly for 48 hours. On analysis at the end of the experiment there was recovered 0.1140 gram of xanthine. There was no uric acid present, not even enough to give a murexide test.

This experiment corroborates the result of the previous experiment in showing the absence of xantho-oxidase. The presence of only adenase and guanase in the mature placenta, as shown by these experiments, does not indicate the probability of any vicarious purine metabolism being performed by the placenta for the fetus, since at this time the fetus is already equipped not only with adenase and guanase, but also with xantho-oxidase.

#### SUMMARY.

The several enzymes which accomplish purine metabolism in the human organism are developed independently as to time during the course of intrauterine life, the fetus at term being equipped with the same enzymes as the adult, thus differing from the dog and pig.

*Guanase* is present in the fetus at the third month, the earliest stage studied, and at all later periods.

*Adenase* is absent in the three-months fetus, but is present at

the fifth month, intermediate stages not having been examined. This demonstrates that these two amidases are not only independent enzymes, but that they are developed independently.

The liver of the human fetus at and after the fifth month contains both adenase and guanase, thus differing from the dog and pig liver.

*Xantho-oxidase* is demonstrable in the liver, and the combined viscera at full term, but not in the muscle, intestine, kidneys, spleen and thymus. It cannot be demonstrated in the liver or other tissues at or before the sixth month, presumably being developed between that time and full term.

Evidence of the lability of xantho-oxidase is obtained, it being destroyed by long standing, or when tissues other than the one containing the xantho-oxidase are present in an autolyzing mixture.

At no time in intrauterine life, or afterwards, does the human tissue seem to have active uricolytic properties.

Fetal tissues seem to contain much more guanine than adenine, since upon autolysis the resulting solution is constantly found to contain about twice as much xanthine as hypoxanthine.

Human placenta contains about 14 per cent of nitrogen (dry weight) and about 2.1 per cent of this is purine nitrogen; that is, about 0.13 per cent of the fresh weight or 0.66 per cent of the dry weight consists of purines. Of the purines approximately 45 per cent is guanine, 40 per cent adenine, and 15 per cent hypoxanthine. A trace of xanthine may also be found, but the amount is so small that it probably is the result of either post-mortem autolysis or of autolysis in infarcted areas.

The presence of xantho-oxidase or uricase could not be demonstrated in mature placenta.





## SOME OBSERVATIONS ON THE STUDY OF THE INTESTINAL BACTERIA.

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The alimentary canal may be regarded from the point of view of bacterial processes within it, as a singularly perfect incubator; an incubator in which there is provided at different levels such a range of reaction and diversity of food that not only are the conditions suitable for the growth of the normal habituated intestinal bacteria but often also for those organisms, capable of developing at body temperature, which are ingested with the food of the host.

An idea of the truly enormous daily bacterial proliferation which takes place in the intestinal tract may be obtained if one remembers that a considerable portion of the fecal mass is made up of the bodies of bacteria, dead and living. At the same time the multiplicity of types and variety of physiological requirements of this intestinal flora are indications of the excellence of the incubator and a strong reminder of the influence which the unrestrained activity of these organisms might conceivably exercise upon the general condition of the host.

The possibilities of bacterial invasion through the intestinal portal of entry have not been overlooked by investigators, and, indeed, among the most brilliant chapters of medicine are those concerning the etiological relationships which have been demonstrated between certain pathogenic bacteria of exogenous origin and specific diseases of the intestinal tract, for example, typhoid, cholera and dysentery.

The very importance of these discoveries has been a potent factor in diverting attention from the studies of the normal intestinal flora with its wealth of problems relating to the principles which govern the activity of these bacteria. Even at the present time the sequence of events which permits the establishment of

these exogenous invaders in the alimentary canal and the exact conditions through which they are able not only to extend and maintain themselves but even to replace wholly or in part the normal flora, are unknown.

It is possible to trace the influence of these epoch-marking studies in the subsequent history and development of Intestinal Bacteriology.

It appears to be a fact that the majority of bacteria of exogenous origin, pathogenic for man (excluding the anaërobes) are relatively inert from the standpoint of chemical activity. On the other hand, these organisms grow in more or less distinctive ways in artificial media, and, usually, they may be recognized by their cultural aspect, their inability to bring about deep-seated changes in their nutrient environment, through specific serum reactions or by their power to initiate characteristic lesions in susceptible animals. In these respects these exogenous organisms contrast in a noteworthy manner with many prominent types of the normal intestinal bacteria.

The more prominent of the latter are distinguished by their chemical or physiological activity and their identification depends far more upon their ability to bring about well-marked chemical changes in their nutrient environment than upon their cultural properties or serum reactions.

The lack of appreciation of this fundamental difference which exists between the relatively inert pathogens and the chemical activity of the more important types of the normal intestinal flora, together with the notoriety that attaches to the former, explains the unprogressive attitude which has characterized many researches on intestinal bacteriology.

While it must be admitted that the purely academic methods of research have resulted in scores of more or less complete morphological and cultural descriptions of bacteria of intestinal origin this knowledge is fragmentary and unclassified. It is devoid of data which would permit one to correlate the presence of these organisms with the diet or condition of the host, or even to form a judgment concerning their numerical relations with other intestinal organisms.

This "bacteriocentric" conception is not illogical when one is dealing with the exogenous pathogens mentioned above, but

it is unproductive of definite results when it is applied in its unmodified form to the study of the normal intestinal flora. It is becoming more and more evident that the problem of intestinal bacteriology must be approached from the dynamical rather than from the cultural standpoint.

Dr. Theobald Smith<sup>1</sup> has stated the case admirably in the following terms: "It is what bacteria do rather than what they are that commands attention, since our interest centers in the host rather than in the parasite."

It is the purpose of this paper (having called attention to the inadequacy of purely academic methods) to indicate in a general way the procedures and use of media through which one may obtain a more comprehensive idea of the significance of bacterial activity in the intestinal tract. For the sake of simplicity, it will be assumed that the host is an experimental animal (preferably a monkey, since its physiology more nearly approaches that of man) under absolute control. Its diet can be regulated at will and its excretions, particularly the urine and feces, can be collected in an uncontaminated state. The diet of this animal may be either purely protein in nature (e. g., hard boiled eggs) or may be carbohydrate. For the latter it has been found that milk with some added dextrose is excellent. This combination contains considerable protein, but, as has been shown in a previous communication,<sup>2</sup> the flora developed is acidophilic and not proteolytic in nature. It should be stated parenthetically that a diet consisting wholly of carbohydrate would be less suited for bacterial development since bacteria need some nitrogen in their food.

The host (monkey) is fed daily with protein or carbohydrate, as outlined above. As this food passes through the alimentary canal from mouth to anus, it is subjected to the action of ferments elaborated by the host. Also, at different levels of the tract it is decomposed in part by various types of bacteria. The predominating types of bacteria which take part in this decomposition are determined largely by the nature of the diet.

When a change is made in the animal's diet from protein to

<sup>1</sup> Theobald Smith: Some Problems in the Life-history of Pathogenic Microorganisms, *Amer. Med.*, viii, pp. 711-718, 1904.

<sup>2</sup> Kendall: This *Journal*, vi, pp. 257-269, 1909.

carbohydrate, or the reverse, it would seem at first sight that two possibilities exist with respect to the behavior of the bacterial flora towards these alterations in pabulum. First it would appear that the types represented might undergo relatively little change, owing to the fact that they accommodate their metabolism to either form of diet; or, secondly it is conceivable that there might be a shifting of the dominant organisms so that upon the protein diet the proteolytic bacteria will be prominent, while acidophilic bacteria<sup>1</sup> will become dominant as the carbohydrate is increased.

Previous experiments<sup>2</sup> have shown that the latter possibility is the one most commonly realized, namely that there is a parallelism between the nature of the diet and the character of the bacterial types represented in the intestinal and fecal flora. Definite evidences of this activity of the intestinal flora are not wanting. In the excretions (particularly in the urine) there occur substances which are the products of bacterial metabolism. These end products of bacterial digestion may be burned in the body, excreted direct, or combined with some substance or substances elaborated by the host to render them less toxic and excreted in this combined form. The presence of these metabolic products in the urine is influenced by two principal factors—bacterial activity and intestinal absorption. Other considerations enter into the problem, and for this reason the qualitative, rather than the quantitative estimation is all that is to be considered in this connection. It follows that the recognition of these end products, *which are in reality indicators of certain definite types of bacterial activity*, is of the greatest importance. Hitherto this correlation between diet, intestinal flora and end products has been largely overlooked, and the natural result has been that the corroborative evidence which these indicators furnish has not been utilized.<sup>3</sup>

<sup>1</sup> For a discussion of the bacterial changes associated with a change in diet see Kendall: *This Journal*, vi, pp. 266–268, 1909.

<sup>2</sup> See Kendall: *loc. cit.*

<sup>3</sup> It is undoubtedly true that some connection between diet and bacteria on the one hand, bacteria and end products on the other, has been surmised, but the lack of definite information available at the present time is strong evidence of the truth of the assertion that the *three* phases—diet, bacteria and end products—have not been considered in their interdependent relations.

Having determined by experiment that a given diet (for example, simple protein) is associated with a definite type of bacterial activity, and that coincidentally certain of these indicators are present in the urine of the host, it becomes a relatively simple matter to isolate individual strains of this fecal flora which will reproduce, either alone or symbiotically with other strains, these same end products. This is accomplished by growing the mixed fecal bacteria in media of the *same fundamental composition* as that of the diet which originally nourished them. An enrichment of the dominating types usually takes place, the abrupt change from intestine to media, with its resulting lack of development is partially overcome, while every possible opportunity is given for the selective development of the desired types. Thus the plating, which must be relied upon for the final separation of the cultures in a state of purity, is far more successful than when plating direct, without the preliminary enrichment, is resorted to. These end products, then, become the criteria through which it is possible to decide with definiteness the participation, indifference or antagonism of each of these types of bacteria in the process under consideration.

In the present undeveloped state of the subject it will be impossible to formulate a definite procedure applicable to all cases. It is very probable, indeed, that from the nature of the phenomena involved, such an undertaking would be disappointing in its results. The best that can be done will be to outline the course of a definite experiment, indicating the procedures through which it is possible to arrive at the desired conclusions.

Before this is done, however, I wish to mention briefly those developments and extensions of present methods which have made it possible to bring the work to its present state. They are: the association of certain products of bacterial metabolism (which are present under specific conditions in the urine of the host) with the activity of certain definite types of organisms upon definite foodstuffs; the corroborative use of artificial media for the demonstration of the completeness and direction with which the bacterial complex in the intestinal tract follows the changes in the character of the diet, and the employment of these media for the selective enrichment and isolation of those varieties of organisms which are most intimately concerned in these changes and the elaboration of these end products.

The following experiment which was repeated several times, always with the same results, may be quoted to demonstrate the general procedure followed in this work.

A monkey was placed upon a diet consisting of milk plus dextrose. Bacterially considered, this diet was essentially carbohydrate in character—there were very few proteolytic bacteria present in the fecal flora, which was of the acidophilic type; the fecal bacteria which developed on this regimen grew less readily in artificial media than was the case with either a mixed or a protein diet.

The acidophilic nature of the fecal flora was brought out in a striking manner by inoculating with the mixed fecal flora milk fermentation tubes,<sup>1</sup> broth fermentation tubes containing dextrose, lactose and saccharose, gelatin and a series of dextrose broth containing varying amounts of acetic acid.<sup>2</sup>

The milk tubes showed coagulation, but no further action was apparent. In the broth fermentation tubes there were slight turbidities with very little or no gas. The gelatin tubes contained only a very slight growth after many days, while the acid dextrose tubes showed moderate development, even in the highest acidities. The milk and gelatin are particularly noteworthy. Milk, and to a lesser extent, gelatin, are excellent media for the development of proteolytic bacteria, while the acidophilic flora grow much less readily on artificial media than do the protein bacteria. Hence the lack of bacterial development in these media is the strongest evidence of the inhibition, or *even replacement of proteolytic organisms* by the acidophilic flora.

The urine was found to be free from indican, phenolic bodies and other products of intestinal putrefaction.

The animal was then placed upon a purely protein regimen with an ample allowance of water.

The conditions changed rapidly. The milk fermentation tubes became the seats of great bacterial activity when they were inoculated with the mixed fecal flora. The milk was greatly peptonized and much gas was formed; liquefaction was marked in the gelatin tubes (stab inoculations); the fermenta-

<sup>1</sup> Theobald Smith, Herbert R. Brown and Ernest L. Walker: *Jour. Med. Research*, xiv, pp. 193-206, 1905.

<sup>2</sup> Hayem's solution. See Finkenstein: *Deutsch. med. Woch.*, p. 263, 1900.

tion tubes showed large amounts of gas (even 90 and 100 per cent being not infrequently produced in eighteen hours); while there was a gradual diminution in the acidophilic flora grown in acid broth tubes. This diminution was manifested chiefly by the inability of the organisms to grow in the highest acidities. These growths took place very rapidly, eighteen to twenty hours being ample time for the described phenomena to develop in their completeness.

Coincidentally products of the decomposition of protein began to appear in the urine. Indican and phenolic bodies were particularly sought for and found in increasing amount as the proteolytic flora became established. Urorosein was not found.

The replacement of the acidophilic flora, then, was demonstrable in the following manner:

a. There was a *microscopical* change in the fecal flora. The strongly Gram-positive fields, consisting largely of the medium sized rod-shaped, acidophilic organisms were replaced by large, Gram-positive rods; smaller, Gram-positive and Gram-negative rods [subtiloid bacilli]:coccal forms in small numbers, and oval, Gram-negative bacilli, referable morphologically to *B. coli* and related aërogenic bacilli.

b. Culturally, whereas the acidophilic (carbohydrate) flora grew very poorly or not at all in gelatin; slowly, with at most coagulation, in milk; moderately, with little or no gas in fermentation media; and considerably in even the highest acidities ( $\frac{N}{10}$ ) in dextrose broth, the exact reverse was the distinguishing feature of the protein diet. Gelatin was promptly liquefied; peptonization and considerable gas-production were features of the milk tubes; heavy turbidities and large volumes of gas were produced in dextrose, lactose and saccharose, while the higher acidities of the acid dextrose bouillon cultures were devoid of growth.

c. *Chemically*, on a carbohydrate diet, with the resulting acidophilic flora, the urine was free from products of intestinal putrefaction. As the protein regimen was established and the proteolytic bacteria became habituated to the changed conditions in the intestinal tract, indican and phenolic bodies gradually became prominent in the urine.

It will therefore be seen that through the use of this general procedure it is possible to demonstrate perfectly definite, con-

sistent correlations between the nature of the diet, the morphology, cultural and physiological relations of the intestinal flora, and the type of and products of bacterial metabolism on each of these diets. These relations are distinctive and sharply defined.<sup>1</sup>

#### SUMMARY.

The procedures in this paper are outlines of general principles applicable to the determination of the more important types of bacterial activity in the intestinal tract and for the isolation of the principal agents concerned in these processes, rather than specific methods to meet special cases. An extension of these principles, along appropriate lines, however, will furnish a definite line of approach to the study of the majority of problems relating to the intestinal flora.

These procedures are based upon the correlation which exists between diet, bacterial flora and end products of bacterial activity which appear in the urine. The nature of the diet practically determines the dominant types of intestinal bacteria, and these organisms in turn, acting upon the digestive products of the diet elaborate the end products of their activity which appear in the urine.

With the exception of a few anaërobes (which derive their oxygen from the combustion of carbohydrates) the majority of the prominent types of the normal flora which develop on a protein diet grow luxuriantly in media free from carbohydrate, while those developing on a carbohydrate regimen grow poorly, or even not at all, unless carbohydrate is present. Hence by inoculating portions of the mixed fecal flora with gelatin and milk and observing the degree and rapidity of peptonization, it is possible to form a judgment of the character of the proteolytic flora. At the same time these media furnish conditions so favorable for the growth of these organisms that they can be regarded as selective for the isolation of the proteolytic flora.

On the other hand, through the use of media containing carbohydrate and particularly the acid dextrose broth, one obtains a

<sup>1</sup> A detailed account of these experiments carried out on monkeys, using these procedures, will be published later.

fairly specific enrichment of the acidophilic flora, characteristic of a carbohydrate regimen.

Furthermore, through the use of these selective media it is possible to form a judgment of the completeness of the bacterial response to the nature of the diet. For example, if the experimental animal is on a carbohydrate regimen, the presence or absence of growth in protein media will indicate the presence or absence of proteolytic bacteria, since the acidophilic organisms do not grow well in these media and cannot, therefore, inhibit the growth of these organisms. Conversely, with a protein diet, the presence or absence of acidophiles may be determined by inoculating the mixed fecal flora into acid dextrose broth, which is unfavorable for the development of the proteolytic types. These determinations may be made roughly quantitative for the different types by inoculating definite amounts of the mixed fecal flora into appropriate media.

The end products of bacterial activity which appear in the urine are important for two reasons: they indicate the types of bacterial activity in the intestinal tract, and their reproduction in artificial media by pure cultures derived from the intestinal flora furnish strong presumptive evidence of the participation of these organisms in the process.



## THE TRANSMISSION OF ACUTE POLIOMYELITIS TO MONKEYS \*

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SIMON FLEXNER, M.D., AND PAUL A. LEWIS, M.D.  
NEW YORK

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Poliomyelitis or infantile paralysis prevailed in epidemic form along the Atlantic seaboard in the summer of 1907. About that time it appeared in Austria and Germany. In the summer of 1909 the disease reappeared as a focalized epidemic in Greater New York and had, by that time, spread widely throughout the United States and Europe.

The cause and mode of dissemination of the disease are unknown; and hence there exists no intelligent means of prevention. While the severity and fatality of the disease fluctuate widely, its effects are always so disastrous as to make it of the highest medical and social importance.

In spite of many thorough studies of the spontaneous disease in man, our knowledge of causation and prevention has not been advanced; it may be hoped that it will be advanced by the opportunity for fundamental study opened up by the successful transmission of the disease to lower animals.

In May, 1909, Landsteiner and Popper<sup>1</sup> published a report of two successful inoculations of monkeys with the spinal cord obtained from two fatal cases of poliomyelitis. The injections were made into the peritoneal cavity. One monkey became paralyzed in the lower extremities and died on the sixth day after inoculation; the other was killed on the nineteenth day. In both, lesions of the spinal cord similar to those in man existed. The disease could not be transferred to other monkeys. Our efforts to transmit the disease to lower animals were first made in 1907, at which time cerebrospinal fluid

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\* From the Laboratories of the Rockefeller Institute for Medical Research.

1. Landsteiner and Popper: *Ztschr. f. Immunitätsforsch.*, Orig., 1909, ii, 377.

obtained by lumbar puncture was introduced into the spinal canal and peritoneal cavity in monkeys and other animals. We were limited to this fluid, as we did not secure material from a fatal case. The results were negative. Since September of this year we have secured suitable material from two cases of poliomyelitis in human beings. For the material from one we are indebted to Dr. Ridner, of Lake Hopatcong, N. J., and for the other to Dr. Le Grand Kerr, of Brooklyn.

Dr. Ridner's patient died on the fifth or sixth day after the appearance of the paralysis, which affected the lower extremities. The lumbar cord was obtained in a sterile condition, twenty-six hours after death, and a portion was inoculated into monkeys about twelve hours later.<sup>2</sup> The entire spinal cord was obtained from Dr. Kerr's case twelve hours after death, and inoculation into monkeys was made four hours later. In Dr. Kerr's case, in which death occurred on the fourth day, the lesions were diffuse throughout the cord. Paralysis had been very extensive. The gross and microscopic lesions were characteristic in both cases.

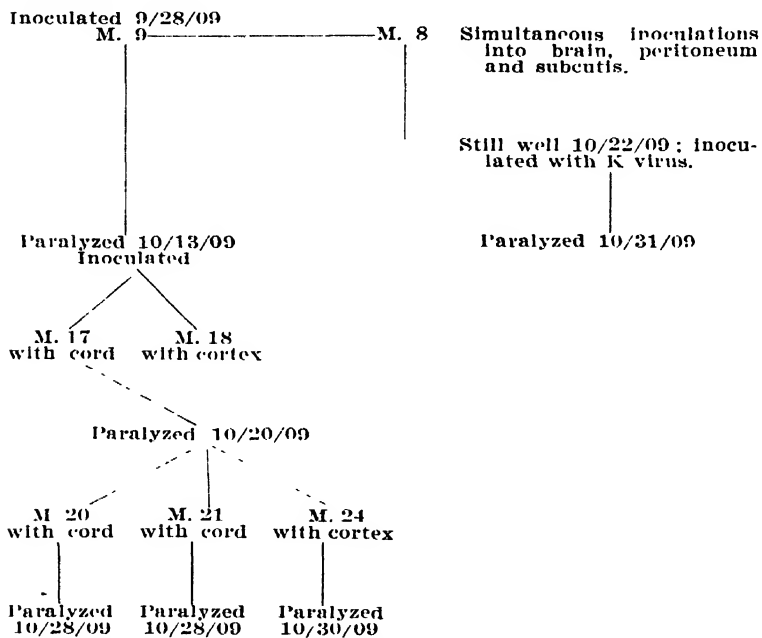
In order to favor the transmission of the disease to monkeys, the brain was chosen as the site of inoculation, which was made under ether anesthesia through a small trephine opening. After the operation, the animals were at once lively and normal. The injected material consisted at first of emulsions in salt solution of the spinal cord from the children and later of emulsions of the spinal cord of monkeys developing the paralysis. An effort was made to enrich the inoculating material by incubating it in celloidin sacs placed in the peritoneal cavity of monkeys and rabbits. At the present time we wish merely to record the series of successful experiments which we have conducted with the spinal cord obtained from the case of Dr. Ridner and designated M. A. The accompanying chart will show at a glance what has been accomplished up to date with the M. A. virus. We may mention here that the microscopic study of the spinal cord from the affected monkeys has shown, without exception, lesions similar to those of poliomyelitis in man. In some cases the lesions in the cords of monkeys could be detected by the naked eye.

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2. No reference will be made in this preliminary report to other varieties of animals employed.

The chart shows unmistakably that by employing the intracranial method of inoculation it is possible to carry the virus of epidemic poliomyelitis successfully through a series of monkeys. It is highly probable that the transmission may be carried on indefinitely. Should this expectation prove well founded, the outlook for securing a fuller understanding of the nature of this disease will be immeasurably improved.

It should incidentally be mentioned that not only is the spinal cord active, but the cortex of the brain also



Transmission of M. A. virus through monkeys. The virus is being transmitted further. The abbreviation M. signifies monkey.

(Monkey 24). A delayed or unsuccessful inoculation may be converted into a successful infection by reinoculation with an active virus (Monkey 8).

It has long been supposed that epidemic poliomyelitis is an infectious disease. Its mode of spread certainly points to that view. A single successful inoculation with human virus could not establish the view, because the result might be due to a transferred toxic body. But now, that successive transfer of the active agent of the

disease has been accomplished, any doubt of its infectious origin can hardly be longer maintained.

The experiments with the virus of poliomyelitis are being continued, as is the search for additional evidences of its micro-organismal nature.<sup>3</sup> The complete protocols of the experiments here summarized and still other experiments will be published in a forthcoming issue of the *Journal of Experimental Medicine*.

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3. A thorough search for bacteria by cultural and other methods was made in 1907, and again this year, but none that could be viewed as the causative agent has been discovered.

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# THE TRANSMISSION OF EPIDEMIC POLIO-MYELITIS TO MONKEYS

A FURTHER NOTE \*

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NEW YORK

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In a previous communication<sup>1</sup> we presented in the form of a chart the results secured up to that time in producing poliomyelitis in monkeys by injecting intracerebrally a virus, denominated M.A., obtained from the spinal cord of a child suffering from epidemic infantile paralysis. The virus had been passed successively through three generations of monkeys and is still being transmitted.

We desire in this communication to present the results in the form of a chart<sup>2</sup> obtained up to this time with the second virus, denominated K. It is, in our opinion, desirable that the facts thus far secured relating to this virus be published, since they extend considerably the previous observations.

In the first place, it can now be affirmed that the virus of poliomyelitis cannot be very difficult of transmission to monkeys under the conditions leading to the development of the lesions and symptoms characteristic of epidemic poliomyelitis in man, since both specimens of human cord furnishing the original virus have sufficed for the transmission of the disease successively.

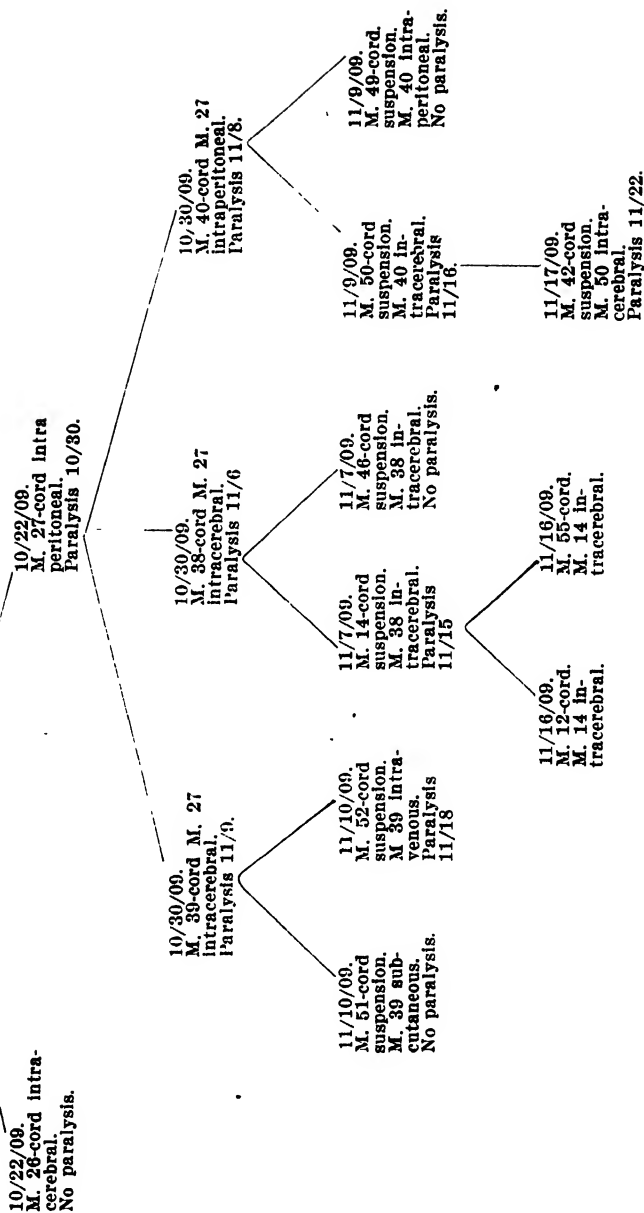
In the next place, it can now be stated that it is not absolutely essential that the virus be introduced into the

\* From the Laboratories of the Rockefeller Institute for Medical Research.

1. THE JOURNAL A. M. A., Nov. 13, 1909, III, 1639.

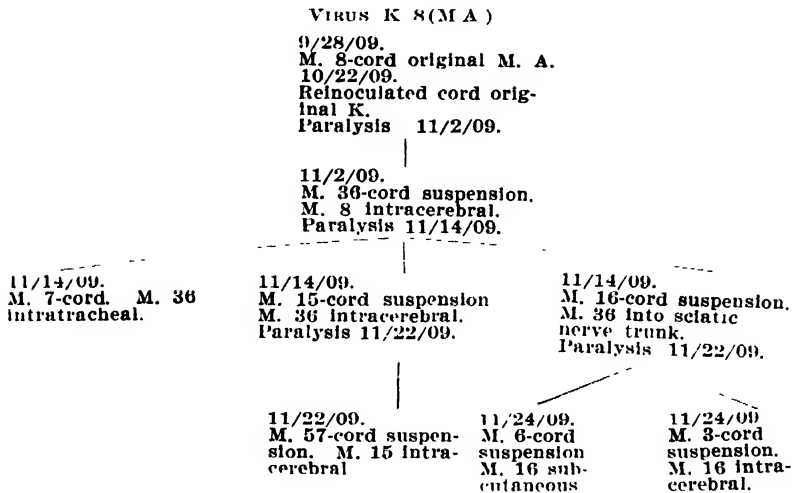
2. In the chart M. signifies monkey.

10/22/09.  
Spinal Cord Case K.



brain, but that successive transmission is possible by way of the peritoneal cavity (Monkeys 27 and 40), by intravascular injection (Monkey 52), and by intraneural injection (Monkey 16). The lesions in the monkey in which the virus was introduced into the sheath of the sciatic nerve developed first on the side inoculated and later extended to the opposite side of the spinal cord.

On the other hand, it cannot yet be affirmed that still other avenues do not exist for the entrance of the virus into the central nervous system. Additional observa-



tions are required and are, indeed, in process of being made, before the statement can be ventured that infection does not occur by way of the skin, the respiratory passages and the digestive tract, and that it may not be accomplished by means of a filtered extract.

The conclusions to be drawn from the experiments related are to the effect that the virus of epidemic poliomyelitis is readily transmissible from man to monkeys and from monkey to monkey, by way of the brain, the peritoneal cavity and the circulation, and that, however transmitted, it becomes established in the spinal cord and medulla, where it sets up characteristic lesions

which are followed by equally characteristic effects that exhibit themselves as the usual symptoms of infantile paralysis in human beings.

Moreover, it can now be stated that this experimental form of poliomyelitis in monkeys is a severe and very often a fatal disease, and when recovery from the disease takes place there persist residues of paralysis which resemble the paralytic effects also persisting in human subjects of poliomyelitis.

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## THE NATURE OF THE VIRUS OF EPIDEMIC POLIOMYELITIS \*

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NEW YORK

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In our previous articles<sup>1</sup> on the transmission of epidemic poliomyelitis to monkeys, we have left undiscussed the nature of the virus responsible for producing the disease. From the beginning, our attention has been directed toward the solution of that fundamental question, but the results of our studies were, until recently, wholly of a negative nature. We failed utterly to discover bacteria, either in film preparations or in cultures, that could account for the disease; and, since among our long series of propagations of the virus in monkeys not one animal showed, in the lesions, the cocci described by some previous investigators and we had failed to obtain any such bacteria from the human material studied by us, we felt that they could be excluded from consideration.

We have, up to this time, made a very painstaking study of film preparations and sections prepared from two specimens of human spinal cord and many specimens of the spinal cord and brain (and other viscera) obtained from monkeys, prepared and stained in the most various ways, but without finding either bacterial or protozoal parasites that could account for the infection.

The readiness with which epidemic poliomyelitis can be transmitted to monkeys and the failure to find visible and stainable parasites in the lesions of the spontaneous and experimental disease led to another line of investi-

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\* From the Laboratories of the Rockefeller Institute for Medical Research, New York.

1. The Transmission of Acute Poliomyelitis to Monkeys, *THE JOURNAL A. M. A.*, Nov. 13, 1909, III, 1639; Dec. 4, 1909, III, 1913.

gation. It is known, for example, that the viruses of vaccinia and rabies, neither of which has been certainly demonstrated in films or sections of tissues or cultivated artificially, withstand very well the action of glycerin, while bacteria withstand it far less well. The bacteria mechanically admixed with the virus of vaccinia can be destroyed by glycerination.

We have, therefore, suspended the comminuted spinal cords of monkeys affected with poliomyelitis in glycerin, and, after an interval of days, we have inoculated the glycerinated virus into normal monkeys. In the experiment to be reported, the cord of Monkey 40, of the second generation of virus K, was kept in glycerin for seven days, after which, the glycerin having been washed away with salt solution, and the suspended tissue recovered by centrifugalization, it was inoculated intracerebrally into Monkey 35. This latter animal developed paralysis on the tenth day after inoculation and showed the characteristic microscopic lesions of epidemic poliomyelitis in the spinal cord and brain.

In order to determine whether this effect was produced by the living virus or by an adherent toxic body, the cord of Monkey 35 was injected into Monkey 58, which developed paralysis eleven days after the inoculation. The lesions in the latter monkey were also characteristic.

The next series of experiments was planned to determine the probable size of the organism producing epidemic poliomyelitis, so far as this could be accomplished by the use of mechanical filters. The experiment to be related briefly was made with the cord of Monkey 56, which succumbed to the fifth generation of virus K. The cord was triturated with sterile quartz sand, mixed with salt solution, thoroughly shaken and pressed through a Berkefeld filter. The clear and bacteriologically sterile filtrate was injected intracerebrally into Monkey 68, which developed paralysis on the seventh day following the inoculation.

From the foregoing experiments, taken in conjunction with the negative results of bacteriologic and histologic examinations, it would appear that the infecting

agent of epidemic poliomyelitis belongs to the class of the minute and filterable viruses that have not thus far been demonstrated with certainty under the microscope.

Since the publication of our last note, the fact has been determined that the virus of poliomyelitis can be transferred to the central nervous system by way of the subcutaneous tissues in monkeys. The two viruses have now each been passed through six series of animals.

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# EPIDEMIC POLIOMYELITIS IN MONKEYS \*

## FOURTH NOTE

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SIMON FLEXNER, M.D., AND PAUL A. LEWIS, M.D.  
NEW YORK

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In our previous communications on experimental epidemic poliomyelitis<sup>1</sup> we described its successive transmission through two series of monkeys and discussed the nature of the virus that causes the disease. In the first place, we stated that by employing the intracerebral mode of inoculation of the virus the disease could be readily transmitted from monkey to monkey, possibly through an indefinite series, but that successive transmission could be accomplished also by means of inoculations into a large nerve (sciatic), into the circulation, the peritoneum and the subcutis. In the second place, we showed that the virus is filterable through a Berkefeld filter and withstands glycerination. In the present article we wish to describe certain additional facts that have been ascertained concerning the virus of epidemic poliomyelitis.

The virus was shown, by inoculation experiments, to be contained in the spinal cord and brain, but it is not known and it has yet to be determined whether it is present in the blood and other organs. We have produced the disease in a monkey by injecting an emulsion of the regional (axillary and inguinal) lymphatic glands, communicating with a nodule set up by a subcutaneous injection of virus, that had induced paralysis. Two other monkeys were inoculated at the same time, one from the spinal cord and the other from the local subcutaneous lesion. The former developed paralysis and the latter is still well.

\* From the Laboratories of the Rockefeller Institute for Medical Research, New York.

1. THE JOURNAL A. M. A., Nov. 13, 1909, III, 1639; Dec. 4, 1909, III, 1913; Dec. 18, 1909, III, 2095.

The degree of resistance of the virus is being studied. It has been determined that the spinal cord from a human case (K.) of poliomyelitis retains its virulence, apparently unimpaired, on being kept constantly frozen at  $-2^{\circ}$  to  $-4^{\circ}$  C., in the Frigo apparatus, for a period of at least forty days; and also when kept for at least fifty days at a temperature about  $+4^{\circ}$  C., during which time the latter specimen of spinal cord became slowly softened through autolysis and overgrown superficially with mold. These experiments have a bearing on the epidemiology of the disease and indicate that the reduction in cases which occurs with the onset of cold weather does not depend upon the destruction of the virus, although it may have to do with an effect on its multiplication.

Moreover, the spinal cord of an affected monkey still transmits the disease, after having been suspended for at least seven days, over caustic potash, in a desiccator.

The activity of filtrates has been confirmed, and the possibility of their action's being due to soluble toxic bodies and not to living organisms has been excluded by transferring the disease by means of the spinal cord obtained from monkeys that succumbed to a filtrate.

*Can the virus be cultivated artificially?* Portions of a bacteria-free filtrate were inoculated into bouillon containing 10 per cent. of rabbits' serum which had been rendered perfectly clear and sterile by being put through a Berkefeld filter; 1 c.c. of a filtrate was mixed with 9 c.c. of the serum bouillon and incubated. On the second day the fluid in the upper half of the tube was cloudy; the turbidity increased, and on the fourth day the fluid was used for inoculation into a monkey which developed paralysis on the thirteenth day. A single loop of this turbid fluid did not set up turbidity in other tubes of the same medium.

A second series of cultivation tests, still in progress, has been carried out with a human ascitic-fluid bouillon made perfectly clear by filtration through Berkefeld filters. Perfectly clear filtrates, prepared from the spinal cord of affected monkeys, added to the ascitic-fluid bouillon, develop turbidity in the thermostat in twenty-four hours or less, and the turbid fluid inoculated into fresh clear tubes of the same medium causes them to become turbid. The clear filtrates do not

produce turbidity, on incubation, in simple bouillon. None of the turbid fluids contained bacteria that could be seen under the microscope or cultivated in nutrient agar, and the dark-field microscope showed no definite bodies.

In a few instances in which parallel injections were made into the subcutaneous tissue and the brain, the monkey inoculated in the first way developed paralysis and the other escaped affection.

*Does an attack of epidemic poliomyelitis that is recovered from afford immunity to reinfection?* Experimental poliomyelitis in monkeys is a very severe disease and, in our series, it has produced death in fully 40 per cent. of the inoculated animals that have developed paralysis. When recovery occurs, residues of paralysis persist; and when death occurs or when the animals are killed some weeks later, atrophy of the gray matter of the spinal cord, corresponding to the paralyzed limbs, is present. We have reinoculated several of the recovered monkeys and have noted in some instances failure of the virus to act while causing paralysis in the control monkeys. A single example will be given.

Monkey 45 was inoculated into the brain on Nov. 6, 1909; November 13, tremulous and sick; November 15, left leg weak. Next day the leg is paralyzed; November 24, general health good, but paralysis persists; November 30, health excellent except for paralysis. On this day reinoculated, together with two controls. Both of the latter became paralyzed, but the reinoculated animal has remained well.

*Can the course of an intracerebral inoculation be modified by the simultaneous injection beneath the skin of a virus altered by heating?* In seeking for facts relating to artificial protection from or resistance to infection, a considerable quantity of an emulsion of active spinal cord, which had been warmed to 55° or 57° C. for one hour, or to 60° C. for half an hour, was injected beneath the skin at the same time that a usual intracerebral injection of virus was given. The two monkeys employed in the experiment developed paralysis in the usual manner.

Brief mention should be made of other species of animals that have been employed for inoculation. Besides many rabbits and guinea-pigs, 1 horse, 2 calves, 3 goats,

3 pigs, 3 sheep, 6 cats, 6 mice, 6 dogs and 4 cats have had active virus introduced into the brain, but without causing any appreciable effect whatever. These animals have been under observation for many weeks.

In the literature on epidemic poliomyelitis in human beings, reference is made to sensory cutaneous disturbances. In every instance in which we have looked for them, we have found lesions similar to those in the spinal cord and brain in the intervertebral ganglia, obtained from the paralyzed monkeys.

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# **EPIDEMIC POLIOMYELITIS IN MONKEYS \***

## **A MODE OF SPONTANEOUS INFECTION**

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**NEW YORK**

In our previous communications<sup>1</sup> on experimental poliomyelitis, we have left entirely undiscussed the manner in which the virus of epidemic poliomyelitis enters and leaves the human body, points which we propose to discuss in the present note. Our studies having supplied the basic fact of the nature of the virus,<sup>2</sup> it became possible to attack the question of the mode of transmission of the spontaneous disease. That epidemic poliomyelitis is an infectious disease has long been suspected, but that it is also a highly contagious one is far less generally admitted. And yet the studies on its epidemiology by Wickman<sup>3</sup> lend considerable support to the latter view.

In the first place, we wish to draw attention to the frequently observed fact that in point of distribution epidemic poliomyelitis resembles epidemic cerebrospinal meningitis. The two diseases, indeed, present, in this respect, such close similarities that they have often been confounded with each other. The chief and striking difference between them relates to the seasonal prevalence, which for epidemic poliomyelitis is midsummer, and for

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1. Flexner and Lewis: *THE JOURNAL A. M. A.*, 1909, llii, 1639; 1909, liii, 1913; 1909, liii, 2095; 1910, liv, 45.

2. Landsteiner and Levaditi (*Compt. rend. Soc. de biol.*, 1909, lxxvii, 592) independently ascertained the filterability of the virus.

3. Wickman: *Beiträge zur Kenntniss der Heine-Medinschen Krankheit*, Berlin, 1907.

epidemic cerebrospinal meningitis, late winter or early spring. Furthermore, the two diseases occur by preference in the same age groups, being most frequent in infants and young children, although not sparing older children and adults, and they present about the same ratio of attack. In the majority of instances a single case appears in a family or home, but often two cases and less often three and more cases appear. The relation between the grouped cases in a house or locality has, in respect to both diseases, been made out only recently by finding in the instance of cerebrospinal meningitis definite diplococcus-carriers, and in poliomyelitis by discovering abortive cases which would seem to act in the manner of microbe-carriers.<sup>3</sup>

It is held that *Diplococcus intracellularis* passes into the cerebrospinal membranes by way of the lymphatic connections existing between them and the nasopharyngeal mucous membrane. It is difficult, if not impossible, to establish, in human beings, the fact that the diplococcus passes from the membranes, by a reverse lymph-current, into the nasopharynx. And yet such a migration is not only highly probable, but would most readily and satisfactorily explain the intracellularis infection of these mucous membranes, which occurs in epidemic cerebrospinal meningitis. In the monkey,<sup>4</sup> infected with *Diplococcus intracellularis* by injection of cultures into the lumbar spinal canal, the migration into the nasopharynx of the diplococcus, contained in leucocytes and free also, has been followed with the microscope. So that the nasopharynx may be viewed both as the site of escape and of entrance of *Diplococcus intracellularis* in man.

We have studied the mucous membrane of the nasopharynx with reference to the virus of poliomyelitis. The entire mucosa of these parts, in monkeys recently paralyzed, has been excised, rubbed up with quartz sand, pressed through a bacteria-tight Berkefeld filter, and injected in the usual manner into the brain of monkeys.

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4. Flexner: Jour. Exper. Med., 1907, ix, 142.

By employing this method, we have been able to produce paralysis and thus to prove that the mucous membrane contains the virus of poliomyelitis. We have also found that when the virus is injected into the spinal canal by lumbar puncture it sets up the disease and causes the characteristic paralysis. The cerebrospinal fluid removed from monkeys at the onset of paralysis is altered: it contains an excess of proteid and lymphocytes and coagulates spontaneously. Paralysis also follows the inoculation of this fluid into the brain.

These experimental results show that a path of elimination of the virus of poliomyelitis is by way of the nasopharyngeal mucosa and indicate that the same path may be traversed in the course of infection. Hence it would seem desirable, at the present stage of our knowledge, to deal prophylactically with epidemic poliomyelitis, as with epidemic cerebrospinal meningitis, by disinfecting and destroying the secretions of the nasal and buccal cavities.<sup>5</sup> The action of disinfecting agents on the virus of poliomyelitis is being studied.

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5. Levaditi and Landsteiner (Compt. rend. de Soc. de biol., 1909, lxxvii, 787) found the salivary glands to contain the virus.



## THE ELIMINATION OF TOTAL NITROGEN, UREA AND AMMONIA FOLLOWING THE ADMINISTRATION OF SOME AMINOACIDS, GLYCYLGLYCIN AND GLYCYLGLYCIN ANHYDRID.

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THE present investigation represents a continuation of a work, the results of which were recently reported by Levene and Kober.<sup>1</sup> In that communication attention was called to the observations made by Graffenberger,<sup>2</sup> Falta,<sup>3</sup> Voigt<sup>4</sup> and others, on the behavior of various aminoacids in the organism. Levene and Kober pointed out that the rate of elimination of the nitrogen introduced into the gastrointestinal tract in the form of protein differs with the character of the protein. It was also pointed out that no attempt had been made to offer a satisfactory explanation for the difference in the resistance shown by individual proteins to the action of digestive glands and organs. On the other hand, it has become known that proteins differ one from another either by the character of the aminoacids which enter into their molecule, or by the mode of union of these acids within the molecule. It was therefore deemed necessary to precede the analysis of the factors regulating the metabolism of individual proteins by the study of the rate of catabolism of simple aminoacids on one hand and of peptids on the other.

The first communication contained a report of the results of the study of the elimination of total nitrogen, of urea and of ammonia nitrogen after administration of two aminoacids and one diketopiperazin, as com-

<sup>1</sup> LEVENE and KOBER: This journal, 1909, xxviii, p. 324.

<sup>2</sup> GRAFFENBERGER: *Zeitschrift für Biologie*, 1891, xxviii, p. 318.

<sup>3</sup> FALTA: *Deutsche Archiv für klinische Medicin*, 1904, lxxxi, p. 231; 1906, lxxvi, p. 517.

<sup>4</sup> VOIGT: *HOFMEISTER's Beiträge*, 1906, v, p. 409.

pared with the results obtained after administration of protein. In the present investigation the number of aminoacids employed was greatly enlarged, the experiment with glycylglycin anhydrid was repeated, and the result compared with that obtained after administration of the peptid. Besides, the plan of the experiment was to a certain degree modified in the present work. In the previous experiments the entire daily ration was given to the animal in one meal, in the morning. This led to a variable rate of nitrogen elimination during the following twenty-four hours. The rate was expressed in the form of a curve, the highest point being reached about six hours after the intake, and then gradually declining. The aminoacid or other substance under investigation was added to the meal. Also on such days the rate of elimination could be presented in the form of a curve. The comparison of the two curves offered, however, certain inconveniences. Furthermore, the large mass of the intake lowered the rate of absorption of the ingested material, and thus somewhat obscured the process as it would have taken place under entirely normal conditions.

For these reasons it was deemed advisable to divide the daily rations into five equal portions, and administer them at regular intervals of three hours each. In this manner there was obtained an approximately uniform nitrogen elimination during all hours of the day, and the rate of elimination on the days of the standard diet could be practically expressed in form of a straight line. The substances added to the standard diet were administered with the morning meal. On such days the rate of elimination was variable during the twenty-four hours following the added intake, and could be expressed in the form of a curve, easily comparable with the straight line of the normal intake.

#### EXPERIMENTAL PART.

**Methods of analysis.**—The methods of analysis employed in these investigations, with one exception, were the same as those described in the previous communication. For the estimation of urea, use was made of the Benedict-Gephart method.<sup>5</sup> The urine was obtained by catheterization at three-hour intervals, and the quantity obtained in

<sup>5</sup> BENEDICT and GEPHART: *Journal of the American Chemical Society*, 1908, **xxx**, p. 1760.

this manner added to any urine voided by the animal in the cage between catheterizations.

**Period of standard diet.** — Three dogs were used in course of this investigation. They will be referred to as dogs A, B and C. The standard diet of dog A varied somewhat in the course of the experiments. It consisted either of:

		gm. N.	calories.
I.	Plasmon . . . . . 17.5 gm. containing	1.99	72
	Cracker meal . . . 100.0 gm.     "	1.50	440
	Lard . . . . . 25.0 gm.     "	...	232
	Total intake . . . . .	3.49	714
II.	Plasmon . . . . . 25.0 gm. containing	2.85	102.5
	Cracker meal . . . 75.0 gm.     "	1.12	307.5
	Lard . . . . . 25.0 gm.     "	...	232.0
	Total intake . . . . .	3.97	642.0

The diet of dog B consisted of:

Plasmon . . . . . 12.5 gm. containing	14.2	51.2
Cracker meal . . . 60.0 gm.     "	0.90	246.0
Lard . . . . . 25.0 gm.     "	...	232.0
Total intake . . . . .	2.32	529.2

The diet of dog C consisted of:

Plasmon . . . . . 16.5 gm. containing	1.880	67.8
Cracker meal . . . 75.0 gm.     "	1.125	307.0
Lard . . . . . 25.0 gm.     "	...	232.0
Total intake . . . . .	3.00	606.8

Tables I, II and III contain the rate of elimination of total nitrogen, of urea and ammonia nitrogen by these two animals on the respective diets. The rate of elimination during the twelve hours beginning with the second three-hour period and ending with the fourth period in which the most marked changes after additional feeding are to be expected, present only very moderate fluctuations. In some other animals used for other experiments the fluctuations were still more insignificant. The protein absorption from the intestinal tract was normal, and the nitrogen distribution in the urine in harmony with previous experience.

*Increased plasmon experiment.*—This experiment was performed on dog B. On the day of the experiment 1.79 gm. of nitrogen in the form of plasmon were added to the standard diet. The rates of elimination through the urine are recorded in Table IV.

	Total N.	Urea N.
The total elimination on the day of experiment .	2.989 gm.	2.684 gm.
The total elimination on the day of standard diet	<u>2.077 gm.</u>	<u>1.792 gm.</u>
	0.912 gm.	0.892 gm.

Comparing the results of this experiment with those reported in the previous publication,<sup>6</sup> one notes that the rate of elimination is higher and that of retention lower when the daily ration is given in fractional doses. Thus, there was a retention after the first twenty-four hours following the intake of the additional plasmon of only 43.46 per cent of nitrogen in the present experiment and of 74 per cent in the older experiment. The excessive nitrogen was eliminated also in this experiment exclusively in the form of urea.

*Alanin experiment.*—The experiments with this aminoacid were performed on dog B. Observations were made with the optically active, naturally occurring l-alanin and with the inactive (d-l) acid. It has been known from previous observations that after the administration of the optical inactive (d-l) form of aminoacids the antipode to the naturally occurring substance reappears in the urine unchanged.<sup>7</sup> However, it has not been known whether or not the d-alanin is removed completely. The observation recorded in the previous publication, that the nitrogen of nitrogenous substances catabolized in the organism of the dog is removed exclusively in the form of urea, furnishes a method for a quantitative estimation of the part which is catabolized as compared with that which is removed without having suffered deterioration. *A priori*, it does not seem improbable that even optical antipodes of the same acid should be utilized by the organism, and for the following reasons. Observations are recorded that on digestion of protein the d-l form of aminoacids is formed.<sup>8</sup> Since only optically active substances enter into the structure of protein, it is natural to believe that the racemization took place in the process of digestion. On the other hand,

<sup>6</sup> LEVENE and KOBER: *Loc. cit.*, p. 323.

<sup>7</sup> KUTCHER: *Zeitschrift für physiologische Chemie*, 1898, xxv, p. 195.

<sup>8</sup> LEVENE: *Ueber die Verdauung der Gelatine*, *ibid.*

racemization consists in the transformation of an optically active substance into its optical antipode. From this it would follow that the organism is in possession of a mechanism by which it can utilize the optical antipodes to the naturally occurring aminoacids. The results of the present experiments indicate that after the administration to a dog of d-l forms of aminoacids only a part of the optical antipode to the natural form is removed unchanged.

The results of the experiments with l-alanin are recorded in Table V. 13 gm. of alanin containing 2 gm. of nitrogen were added to the standard diet containing 3.97 gm. of nitrogen. The output was the following:

	Total N.	Urea N.
On the day of the l-alanin feeding . . . . .	5.568 gm.	5.182 gm.
On the day of the standard diet . . . . .	<u>3.598 gm.</u>	<u>3.208 gm.</u>
Removed in excess over the day of the standard diet . . . . .	1.973 gm.	1.974 gm.

Thus all the nitrogen of the alanin was removed within the first twenty-four hours after its intake, and of this nearly 90 per cent was removed within the first nine hours. This rate of elimination is much higher than the one observed under the previous mode of experimentation. Also here the entire excessive nitrogen was removed in the form of urea.

The results of the experiment with l-alanin are recorded in Table VI; 20 gm. of alanin containing 3.15 gm. were added to the second standard diet of dog A. The output was the following:

	Total N.	Urea N.
On the day of the experiment . . . . .	6.755 gm.	5.860 gm.
On the day of the standard diet . . . . .	<u>3.595 gm.</u>	<u>3.203 gm.</u>
In excess over the standard diet . . . . .	3.160 gm.	2.657 gm.

The general character of nitrogen elimination was similar to the one after feeding of l-alanin. All the nitrogen of the additional intake was removed within the first twenty-four hours and the larger part of it (nearly 76 per cent) within the first nine hours after the intake.

Of the excessive nitrogen contained in the urine on the day of the experiment only 84 per cent was in the form of urea. Taking into consideration that the ingested i-alanin contained 50 per cent of l-alanin which is completely converted into urea, one reaches the conclusion that of the remaining 50 per cent only 34 per cent was converted into urea

and 16 per cent was removed unchanged, thus showing that of the optical antipode to the natural alanin only 32 per cent was eliminated without having undergone any change.

*Leucin experiment.*—Also with this aminoacid an attempt was made to compare the behavior of the two optical forms, the naturally occurring l-leucin and the d-l leucin. It was found impossible, however, to obtain a successful experiment with the latter form, since dogs invariably vomited after administration of i-leucin. Therefore the experiments were performed only with l-leucin. Only two experiments with this aminoacid are recorded by previous observers, namely, by Abderhalden and Samuely.<sup>9</sup> The results of their experiments are not very convincing, though the conclusions the authors base on them are correct. In our experiments on dog B, 14.4 gm. of leucin containing 1.54 gm. of nitrogen were added to the standard diet. The results are recorded in Table VII. The output was the following:

	Total N.	Urea N.
On the day of the experiment . . . . .	2.899 gm.	2.614 gm.
On the day of the standard diet . . . . .	<u>2.077 gm.</u>	<u>1.792 gm.</u>
In excess over the standard diet . . . .	0.822 gm.	0.822 gm.
On the day following the experiment . . . .	2.635 gm.	2.346 gm.
On the day of the standard diet . . . . .	<u>2.077 gm.</u>	<u>1.992 gm.</u>
In excess over the standard diet . . . .	0.558 gm.	0.554 gm.

Comparing the results of these experiments with those when the animals were fed on the lower aminoacids, one is struck by the low rate of nitrogen elimination. Only 53.37 per cent of the excessive intake was removed during the first twenty-four hours, 36.23 per cent of the intake was eliminated in the following twenty-four hours. Thus it may appear as if the administration of this aminoacid may be followed by a lasting nitrogen retention. In order to test the possibility of nitrogen retention after administration of l-leucin, this aminoacid was added continually for five days in quantities containing 1 gm. of nitrogen per day. The results are recorded in the following table, which demonstrates that there was no nitrogen retention following the five days of

<sup>9</sup> ABDERHALDEN and SAMUELY: *Zeitschrift für physiologische Chemie*, 1906, xlvii, p. 346.

feeding with leucin in addition to the standard diet. As will be seen from results to be published in a subsequent paper, this low rate of nitrogen eliminated may be explained by the slow transportation of leucin from the stomach into the intestinal tract, and by the low rate of absorption of the acid through the gastric wall. Comparing the results recorded in Table VII with those obtained on feeding with the lower aminoacids, one notes the slow and continuous rise of the nitrogen output on the day of the experiment as compared with the normal days.

The nitrogen recovered in excess over that of the normal days is composed exclusively of urea, also after feeding of l-leucin.

#### ADMINISTRATION OF L-LEUCIN.

Day.	Total nitrogen in grams.		Day.	Total nitrogen in grams.	
	Urine.	Feces.		Urine.	Feces.
1	2.68	....	4	3.51	0.294
2	2.94	0.520	5	2.41	0.293
3	3.21	0.304	6	2.42	0.286
Total . . . . .				17.17	1.697
Intake . . . . . Food 13.98					
l-Leucin 5.00					
Total . . . . .				18.98 gm. N.	
Output . . . . . Urine 17.17					
Feces 1.70					
Total . . . . .				18.87 " "	
Balance . . . . .				+0.11 gm. N.	

*Phenylalanin experiment.* — On the day of the experiment on dog A, 24 gm. of l-d phenylalanin were added to the second standard diet. The output was the following:

	Total N.	Urea N.
On the day of the experiment . . . . .	5.265 gm.	4.302 gm.
On the day of the standard diet . . . . .	3.595 gm.	3.203 gm.
In excess over the standard diet . . . . .	1.670 gm.	1.099 gm.

The details of the experiment are recorded in Table VIII. The analysis of this table reveals the fact that after administration of this amino-acid the rate of the elimination of the excess nitrogen followed the course of nitrogen elimination after leucin feeding. As compared with the days when the lower aminoacids were added to the standard diet, the nitrogen output presented a slower and a more continuous rise.

Of the excessive nitrogen removed by the urine only 65.8 per cent were in the form of urea. Accepting that the nitrogen of the natural l-phenylalanin is converted completely into urea, one is led to the conclusion that of the optical antipode only 31.6 per cent is converted into urea, the remaining portion is removed unaltered.

*Asparaginic acid experiment.*—In a previous publication Levene and Kober<sup>10</sup> noted that the rate of absorption of asparagin from the gastrointestinal tract and the rate of the nitrogen elimination following its ingestion appeared of lower magnitude, compared with that following the administration of glycine. It is very probable, particularly on the ground of the statements of Osborne,<sup>11</sup> that substances of the chemical nature of asparagin (acid amids) are present in the protein molecule. Through the action of mineral acids and enzymes the amido group of these substances is removed with comparatively little resistance, and the acid amid is transformed into the original acid. On the other hand, the original acid-asparaginic acid in this instance represents a constant component of the protein molecule. In view of all these considerations it was deemed of particular interest to compare the behavior in the organism of asparaginic acid with that of asparagin. The experiment was performed on dog A. 19.0 gm. of d-l asparaginic acid containing 2.0 gm. of nitrogen were added to the first standard diet. The output was the following:

	Total N.	Urea N.
On the day of the experiment . . . . .	5.185 gm.	4.548 gm.
On the day of the standard diet . . . . .	3.380 gm.	3.029 gm.
In excess over the standard diet . . . . .	1.805 gm.	1.519 gm.

The analysis of Table IX reveals a rate of nitrogen elimination not unlike the one following the administration of the lower aminoacids.

<sup>10</sup> LEVENE and KOBER: This journal, 1909, xxiii, p. 332.

<sup>11</sup> THOMAS OSBORNE, C. S. LEAVENWORTH and C. A. BRAUTLECHT: This journal, 1908, xxiii, p. 180.

During the first twelve hours following the intake 86.66 per cent of the excessive output was removed. There was no appreciable retention of nitrogen after the first twenty-four hours.

Of the total excessive output 84 per cent was removed in the form of urea. On the basis that all the nitrogen of the l-asparaginic acid was removed in the form of urea, the conclusion may be reached that of the optical antipode 31.6 per cent was removed unaltered. The same value was found for d-alanin.

*Arginin experiment.*—The experiment was performed on dog B. The arginin employed in the experiment was obtained by the tryptic digestion of edestine. 3.55 gm. of arginin containing 1.142 gm. of nitrogen were added to the first meal. The output of nitrogen was as follows:

	Total N.	Urea N.
On the day of the experiment . . . . .	3.017 gm.	2.706 gm.
On the day of the standard diet . . . . .	2.077 gm.	1.792 gm.
In excess over the standard diet . . . . .	0.940 gm.	0.914 gm.

The analysis of Table X reveals that the rate of elimination of the excessive nitrogen after administration of arginin as compared with the rate following the administration of the lower aminoacids is lower, and the elimination more protracted. This seems rather significant since arginin is a derivative of guanidine and possesses a high solubility.

Only 97 per cent of the excessive nitrogen was removed in the form of urea. This was possibly caused by the fact that the arginin was to some extent racemized.

*Glycylglycin and glycylglycin anhydrid experiments.*—Feeding experiments with mono peptides and their anhydrids had been performed by Abderhalden and Rona.<sup>12</sup> The authors did not record any difference in the behavior of the anhydrids as compared with peptids. On the other hand, in an experiment performed by Levene and Kober<sup>13</sup> the observation was made that the anhydrid was removed through the urine apparently without having suffered any alteration. The experiment had been performed on a dog in a state of inanition and could not be repeated at that time. It was, therefore, concluded to repeat the

<sup>12</sup> ABDERHALDEN and RONA: *Zeitschrift für physiologische Chemie*, 1905, xlv, p. 176.

<sup>13</sup> LEVENE and KOBER: *Loc. cit.*

experiment and to compare the results with those obtained on feeding the peptid.

The experiments were performed on dog B. On the day of the peptid experiment the dog received 7 gm. of glycylglycin containing 1.48 gm. of nitrogen. The nitrogen output was the following:

	Total N.	Urea N.
On the day of experiment . . . . .	3.676 gm.	3.348 gm.
On the day of the standard diet . . . . .	<u>2.077 gm.</u>	<u>1.792 gm.</u>
In excess over standard diet . . . . .	1.599 gm.	1.556 gm.

The rate of nitrogen elimination is recorded on Table XI, and shows great similarity with that following glycin administration. All the excessive nitrogen is removed in the form of urea.

Two experiments were performed with the anhydrid. In each experiment 6 gm. of the substance containing 1.47 gm. of nitrogen were added to the standard diet. The nitrogen output was the following:

	Total N.	Urea N.
First exp. on the day of the experiment . . . . .	3.353 gm.	1.747 gm.
First exp. on the day of the standard diet . . . . .	<u>2.077 gm.</u>	<u>1.792 gm.</u>
In excess over the standard diet . . . . .	1.276 gm.	0.045 gm.
Second exp. on the day of the experiment . . . . .	3.480 gm.	1.771 gm.
Second exp. on the day of the standard diet . . . . .	<u>2.077 gm.</u>	<u>1.792 gm.</u>
In excess over the standard diet . . . . .	1.403 gm.	0.021 gm.

The rate of nitrogen elimination in these two experiments is recorded in Tables XII and XIII, and shows a more rapid increase than the one following the administration of glycin or of glycylglycin. Of the total excessive nitrogen in one experiment 87.77 per cent and in the other 83.86 per cent is removed within the first nine hours after the intake. In neither of the two experiments was any transformation observed of the excess nitrogen into urea. Since after administration of the peptid such transformation does occur, one is justified to conclude that the anhydrid is removed through the urine unchanged.

*Gelatine experiment.* — It seemed possible to base on the property of glycylglycin anhydrid — to pass unaltered through the organism of the dog — a method for ascertaining whether or not the anhydrids of the peptids, or the diketopiperazines enter into the composition of the

protein molecule. *A priori* this seems possible. Existing experimental evidence is, however, not conclusive. With certainty a diketopiperazin was obtained on protein cleavage only once, namely, by Levene and Beatty<sup>14</sup> on tryptic digestion of gelatine.

However, the digestion in that instance was allowed to continue many months, and thus the possibility is not excluded that the transformation of the peptid was a secondary process. On the basis of the experiment with glycylglycin anhydrid one is led to expect that when proteins containing diketopiperazins in their molecule are added to a standard diet of a dog, the excessive nitrogen thus introduced in the organism will only in part be removed in the form of urea.

On the day of experiment the dog C received 14 gm. of gelatine containing 2.00 gm. of nitrogen in addition to the standard diet. The nitrogen output was as follows:

	Total N.	Urea N.
On the day of experiment . . . . .	4.315 gm.	3.960 gm.
On the day of the standard diet . . . . .	2.514 gm.	2.158 gm.
	<hr/> 1.801 gm.	<hr/> 1.802 gm.

It is evident from the figures that all of the excessive nitrogen administered as gelatine is eliminated in the form of urea. Thus, this experiment leads to the conclusion that either diketopiperazins do not enter into the composition of the protein molecule, or that the anhydrids of peptids within the protein molecule offer less resistance than when in a free state. The rate of nitrogen elimination is recorded in Table XV.

<sup>14</sup> LEVENE and BEATTY: Berichte der deutschen chemischen Gesellschaft, 1906, xxxix, p. 2091.

TABLES I-III.

TABLE I. STANDARD DIET A, DOG I.

Periods.	Total nitrogen in grams.	Urea nitrogen.		Ammonia nitrogen.		Undetermined nitrogen.	
		Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.
9-12	0.266	0.235	88.5	0.003	1.1	0.028	10.5
12-3	0.515	0.468	90.1	0.006	1.1	0.041	8.0
3-6	0.595	0.526	88.5	0.018	3.0	0.051	8.5
6-9	0.672	0.591	88.0	0.019	2.8	0.062	9.2
9-12	0.455	0.401	89.2	0.015	3.6	0.039	8.5
12-9	1.092	0.982	89.9	0.068	6.2	0.042	3.9

TABLE II. STANDARD DIET B, DOG I.

9-12	0.427	0.379	88.9	0.017	3.9	0.031	7.0
12-3	0.555	0.511	92.1	0.021	3.8	0.023	4.2
3-6	0.545	0.494	90.6	0.018	3.3	0.031	5.7
6-9	0.512	0.461	89.8	0.016	3.1	0.035	6.8
9-12	0.436	0.395	90.4	0.018	4.1	0.023	5.3
12-9	0.904	0.789	87.0	0.073	8.1	0.042	4.7

TABLE III. STANDARD DIET, DOG II.

9-12	0.135	0.115	85.2	0.006	4.4	0.014	10.3
12-3	0.434	0.368	84.8	0.026	6.0	0.040	9.2
3-6	0.310	0.265	85.2	0.025	8.0	0.020	6.4
6-9	0.344	0.300	88.4	0.028	8.2	0.016	4.6
9-12	0.294	0.255	86.8	0.025	8.5	0.014	4.7
12-9	0.560	0.492	87.6	0.039	6.9	0.029	5.2

TABLES IV-VI.

TABLE IV. STANDARD DIET, DOG C.							
Periods.	Total nitrogen in grams.	Urea nitrogen.		Ammonia nitrogen.		Undetermined nitrogen.	
		Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.
9-12	0.212	0.172	81.2	0.008	3.7	0.032	15.1
12-3	0.397	0.356	89.7	0.011	2.8	0.030	7.5
3-6	0.385	0.337	87.5	0.013	3.9	0.035	9.1
6-9	0.403	0.343	85.1	0.016	3.8	0.044	11.0
9-12	0.362	0.297	81.4	0.010	3.4	0.053	15.2
12-9	0.759	0.653	86.3	0.037	4.9	0.069	8.7

TABLE V. STANDARD DIET AND PLASMON.							
9-12	0.245	0.223	91.1	0.004	1.6	0.018	7.3
12-3	0.532	0.483	90.8	0.014	2.6	0.035	6.6
3-6	0.546	0.490	89.8	0.014	2.6	0.042	7.6
6-9	0.504	0.449	89.1	0.031	6.1	0.024	4.8
9-12	0.308	0.283	91.8	0.011	3.6	0.014	4.6
12-9	0.854	0.756	88.5	0.056	6.5	0.042	5.0

TABLE VI. STANDARD DIET AND L-ALANIN.							
9-12	0.761	0.701	92.1	0.005	0.6	0.055	7.2
12-3	1.092	0.995	91.1	0.017	1.5	0.080	7.3
3-6	1.277	1.188	93.0	0.029	2.3	0.060	4.7
6-9	0.702	0.640	91.2	0.032	4.5	0.030	4.3
9-12	0.616	0.599	97.2	0.008	1.3	0.009	1.5
12-9	1.120	1.059	94.6	0.041	3.6	0.020	1.8

<sup>1</sup> These figures show balance with standard diet.

TABLES VII-IX.

TABLE VII. STANDARD DIET AND L-ALANIN.

Periods.	Total nitrogen in grams.	Urea nitrogen.		Ammonia nitrogen.		Undetermined nitrogen.	
		Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.
9-12	0.980	0.856	87.5	0.009	0.9	0.115	11.5
12-3	+0.714						
	1.557	1.268	81.5	0.022	1.4	0.267	17.1
3-6	+1.042						
	1.224	1.116	91.1	0.036	2.9	0.073	5.9
6-9	+0.630						
	0.885	0.774	87.5	0.031	3.5	0.085	9.0
9-12	+0.213						
	0.899	0.805	90.5	0.040	4.5	0.045	5.0
12-9	+0.435						
	1.218	1.041	85.5	0.079	6.5	0.098	8.0
	+0.126						

TABLE VIII. STANDARD DIET AND L-LEUCIN.

9-12	0.168	0.150	89.2	0.010	5.9	0.008	4.8
	+0.033						
12-3	0.406	0.368	90.6	0.026	6.4	0.012	3.0
	-0.028						
3-6	0.462	0.417	90.3	0.028	6.1	0.017	5.7
	+0.152						
6-9	0.560	0.516	92.2	0.020	3.6	0.024	4.3
	+0.216						
9-12	0.371	0.337	90.8	0.010	2.7	0.024	6.5
	+0.077						
12-9	0.932	0.826	88.6	0.062	6.7	0.044	4.5
	+0.372						

TABLE IX. STANDARD DIET AND L-PHENYLALANIN.

9-12	0.441	0.329	74.8	0.021	4.8	0.091	20.6
	+0.175						
12-3	0.586	0.463	79.1	0.025	4.2	0.098	17.0
	+0.071						
3-6	0.640	0.518	80.9	0.025	3.9	0.097	15.1
	+0.045						
6-9	0.882	0.710	80.6	0.039	4.4	0.033	3.7
	+0.110						
9-12	0.700	0.602	86.1	0.035	5.0	0.063	9.0
	+0.245						
12-9	2.016	1.680	84.1	0.140	6.9	0.196	9.7
	+0.924						

TABLES X-XII.

TABLE X. STANDARD DIET AND L-ASPARAGINIC ACID.

Periods.	Total nitrogen in grams.	Urea nitrogen.		Ammonia nitrogen.		Undetermined nitrogen.	
		Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.
9-12	0.459	0.380	82.8	0.033	7.2	0.046	10.0
12-3	+0.032						
	1.052	0.922	87.6	0.062	5.9	0.068	6.5
3-6	+0.497						
	1.070	0.837	78.2	0.045	4.2	0.188	17.5
6-9	+0.525						
	0.868	0.810	93.4	0.030	3.5	0.028	3.2
9-12	+0.356						
	0.644	0.608	94.5	0.022	3.4	0.014	2.2
12-9	+0.208						
	1.092	0.991	90.7	0.045	4.1	0.056	5.1
	+0.188						

TABLE XI. STANDARD DIET AND ARGININ.

9-12	0.322	0.256	79.6	0.003	0.9	0.063	19.5
12-3	+0.187						
	0.644	0.595	92.4	0.017	2.6	0.032	4.9
3-6	+0.210						
	0.476	0.445	93.4	0.017	3.6	0.014	3.0
6-9	+0.166						
	0.462	0.431	93.2	0.017	3.7	0.014	3.1
9-12	+0.118						
	0.357	0.312	87.4	0.024	6.7	0.021	5.9
12-9	+0.063						
	0.756	0.667	88.2	0.068	9.0	0.021	2.8
	+0.196						

TABLE XII. STANDARD DIET AND GLYCYLGLYCIN.

9-12	0.364	0.345	94.8	0.011	3.0	0.008	2.2
12-3	+0.229						
	1.036	0.954	92.0	0.028	2.7	0.054	5.3
3-6	+0.602						
	0.580	0.534	92.1	0.020	3.4	0.026	4.5
6-9	+0.270						
	0.490	0.446	91.0	0.018	3.7	0.024	5.3
9-12	+0.146						
	0.434	0.395	91.0	0.004	8.9	0.035	8.1
12-9	+0.140						
	0.772	0.674	87.4	0.062	8.0	0.036	4.6
	+0.212						

TABLES XIII-XV.

TABLE XIII. STANDARD DIET AND GLYCYLGLYCIN ANHYDRID.

Periods.	Total nitrogen in grams.	Urea nitrogen.		Ammonia nitrogen.		Undetermined nitrogen.	
		Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.
9-12	0.507	0.158	31.1	0.006	1.2	0.343	67.7
12-3	0.960	0.282	29.4	0.028	2.8	0.650	67.8
3-6	0.532	0.294	55.3	0.012	2.3	0.226	42.5
6-9	0.442	0.259	58.8	0.021	4.7	0.162	36.5
9-12	0.282	0.220	78.1	0.016	5.7	0.078	19.2
12-9	0.630	0.534	84.7	0.042	6.7	0.054	8.6

TABLE XIV. STANDARD DIET AND GLYCYLGLYCIN ANHYDRID.

9-12	0.654	0.209	31.9	0.009	1.4	0.436	66.7
12-3	0.875	0.254	29.0	0.014	1.6	0.607	69.4
3-6	0.524	0.250	47.7	0.030	5.7	0.244	46.6
6-9	0.405	0.287	70.8	0.014	3.4	0.104	25.7
9-12	0.348	0.253	72.7	0.015	4.3	0.080	23.0
12-9	0.674	0.518	76.9	0.069	10.2	0.087	12.9

TABLE XV. STANDARD DIET AND GELATINE.

9-12	0.456	0.407	89.2	0.015	3.3	0.034	7.5
12-3	0.785	0.732	93.3	0.025	3.2	0.028	3.5
3-6	0.787	0.731	92.8	0.023	2.9	0.033	4.2
6-9	0.795	0.733	92.2	0.026	3.3	0.036	4.5
9-12	0.492	0.455	92.6	0.018	3.7	0.019	3.7
12-9	1.000	0.902	90.2	0.053	5.3	0.045	4.5

TABLES I-XV.  
TOTALS FOR TWENTY-FOUR HOURS.

No. of table.	Diet.	Total nitrogen in grams.	Urea nitrogen.		Ammonia nitrogen.		Undetermined nitrogen.	
			Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.	Grams.	Per cent of total nitrogen.
I	Standard I, Dog A (I A) . .	3.595	3.203	89.3	0.129	3.6	0.263	7.3
II	Standard II, Dog A (II A) . .	3.380	3.029	89.6	0.163	4.8	0.185	5.5
III	Standard Dog B (B) . . . .	2.077	1.792	86.5	0.149	7.1	0.133	6.4
IV	Standard Dog C (C) . . . .	2.514	2.158	85.8	0.097	3.7	0.263	9.9
V	B and Plasmon . . . . .	2.989	2.684	89.6	0.134	4.6	0.171	5.7
VI	I A and I-Alanin . . . . .	5.568	5.182	93.0	0.132	2.4	0.254	4.6
VII	I A and i-Alanin . . . . .	6.755	5.860	86.8	0.227	3.4	0.668	9.9
VIII	B and I-Leucin . . . . .	2.899	2.614	90.2	0.154	5.4	0.131	4.5
IX	I A and i-Phenylalanin . . . .	5.265	4.302	81.7	0.285	5.4	0.678	12.8
X	II A and i-Asparaginic-acid . .	5.185	4.548	88.6	0.237	4.5	0.400	7.7
XI	B and Arginin . . . . .	3.017	2.706	89.8	0.146	4.8	0.165	5.4
XII	B and Glycylglycin . . . . .	3.676	3.348	91.1	0.143	3.9	0.185	5.0
XIII	B and Glycylglycin anhydrid	3.353	1.747	52.1	0.125	3.7	1.481	44.2
XIV	B and Glycylglycin anhydrid	3.480	1.771	50.8	0.151	4.3	1.558	44.8
XV	C and Gelatine . . . . .	4.315	3.960	91.8	0.160	3.7	0.197	4.5



**324. P. A. Levene und J. A. Mandel:  
Über die Konstitution der Thymo-nucleinsäure.**

[Aus dem Rockefeller-Institute für Medical Research, New York.]

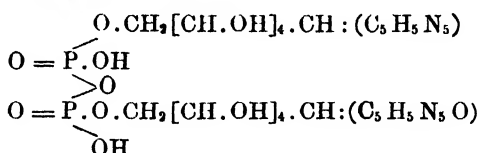
(Eingegangen am 25. Mai 1908.)

Wie bekannt, besteht die Thymonucleinsäure aus Purinbasen, Pyrimidinbasen, Kohlehydraten und Phosphorsäure. Die Art und Weise, in welcher diese Bestandteile mit einander verbunden sind, ist nicht ganz aufgeklärt. Nach den Ansichten von Schmiedeberg<sup>1)</sup> soll ein Komplex Nucleotin existieren, welcher mit Phosphorsäure sich zu einer Nucleotinphosphorsäure verbindet, die dann weiter in eine lockere Bindung mit Purinbasen eintritt und die Nucleinsäure bildet. In der Tat ist es Alsberg<sup>2)</sup> gelungen, Substanzen mit der Zusammensetzung des hypothetischen Nucleotins und der Nucleotinphosphorsäure zu gewinnen. Das Verhältnis des Nucleotins zu den einfachen Bestandteilen der Nucleinsäure ist ohne Aufklärung geblieben. Schon

<sup>1)</sup> Arch. für exp. Path. u. Pharmacol. 43, 65 [1899].

<sup>2)</sup> Ibid. 51, 240 [1904].

vor mehreren Jahren war der eine von uns mit der Untersuchung der intermediären Produkte, welche bei der hydrolytischen Spaltung der Nucleinsäure entstehen, beschäftigt<sup>1)</sup>. Diese Untersuchung wurde fortgesetzt. Die Resultate dieser Untersuchung haben einige Beweise dafür beigebracht, daß die Nucleinsäure aus einfacheren Komponenten zusammengesetzt ist, welche je aus einer Phosphorsäure, einem Kohlehydrat und einer Base bestehen. Diese Komplexe binden sich in der Weise, daß sie eine Polyphosphorsäure bilden. Die Base ist wahrscheinlich mit der Zuckergruppe in glykosidartiger Form gebunden, etwa nach dem folgenden Schema:



Diese Ansicht stützt sich auf die Beobachtung, daß man bei der Spaltung mittels verdünnter Mineralsäure zu Verbindungen gelangt, welche nur noch Spuren von Phosphorsäure enthalten und Kohlehydrate im Molekül haben, aber dabei keine reduzierende Eigenschaft auf Fehlingsche Lösung besitzen. Ferner gewinnt man Substanzen, welche keine Purinbasen mehr enthalten, und bei der weiteren Spaltung Phosphorsäure, Lävulinsäure und Thymin liefern. Nun ist es endlich gelungen, ein Bariumsalz zu gewinnen, welches die Zusammensetzung eines solchen der Glykophosphothyminsäure hatte. Die Substanz war zwar amorph und der Phosphorgehalt wich von der theoretischen Zahl etwas ab, doch stimmten alle anderen analytischen Angaben mit jenen überein, welche durch die genannte Annahme verlangt werden.

Nach dieser Ansicht sind die komplizierteren Nucleinsäuren nach demselben Schema konstruiert, wie die einfacheren, die Guanylsäure und die Inosinsäure. Die Ordnung der Bindung der letzteren ist: Phosphorsäure, Kohlehydrat und Base in glykosidartiger Verbindung. Doch darf man nicht annehmen, daß die Basen mit der Zuckergruppe in den einfacheren Nucleinsäuren (Mononucleotiden) auf dieselbe Weise gebunden sind, wie in den komplizierteren (Polynucleotiden). Während bei der Guanylsäure und der Inosinsäure der freie Zucker oder die reduzierende Glykophosphorsäure schon bei milder Hydrolyse abgespalten werden, ist es überhaupt unmöglich, dasselbe Resultat bei der Spaltung der Thymonucleinsäure zu erreichen. Die Anwesenheit

<sup>1)</sup> Americ. Journal of Physiol. 12, 213 [1904].

der Kohlehydratgruppe kann nur durch Farbenreaktion oder durch Überführen in Lävulinsäure nachgewiesen werden<sup>1)</sup>.

Diese Differenz kann wohl dadurch erklärt werden, daß in den einfachen Nucleinsäuren (Mononucleotiden) der Komplex Zucker-Base durch das Austreten von einem Molekül Wasser entsteht, während er in der Thymonucleinsäure durch das Austreten von zwei solchen Gruppen gebildet ist. Wir werden später Tatsachen erwähnen, die für diese Ansicht sprechen.

Über die Anzahl solcher einfacher Nucleotide, welche bei der Bildung der Thymonucleinsäure sich vereinigen, kann man mit Sicherheit gegenwärtig noch nichts behaupten. Will man der Nucleinsäure die von Stendel angegebene Formel zuschreiben, dann wird sie als Tetranucleotid betrachtet. Die Zahlen, welche der eine von uns (Levene) vor einigen Jahren für die Milznucleinsäure angegeben hat, sprechen aber besser für die Formel  $C_{54}H_{71}N_{20}O_{37}P_5$ <sup>2)</sup>, welche als Pentanucleotid betrachtet werden kann, wobei drei Komplexe durch Austritt von je drei Wassermolekülen gebunden sind.

$C_{54}H_{71}N_{20}O_{37}P_5$ . Ber. C 37.00, H 4.00, N 16.57, P 9.00.

Gef. » 37.78, » 4.86, » 16.00, » 8.91.

### Experimentelles.

80.0 g Nucleinsäure wurden mit 1500 ccm 2-prozentiger Schwefelsäure mit Rückflußkühler im Ölbad von 150° 4 Stunden erhitzt. Es resultierte eine braune Flüssigkeit, fast ohne Rückstand, der so oft bei der Spaltung der Nucleinsäure entsteht. Die Lösung wurde filtriert, mit einem Überschuß von Silbersulfat behandelt und über Nacht im Eisschrank stehen gelassen. Der Niederschlag wurde abfiltriert und das Filtrat mit Barytwasser bis zur alkalischen Reaktion behandelt. Es bildete sich ein zweiter Niederschlag; dieser wurde abfiltriert, vom Silber mit Schwefelwasserstoff und vom Überschuß an Baryt mit Kohlensäure befreit. Das Filtrat wurde bei vermindertem Druck eingedampft, wieder filtriert und mit dem gleichen Volumen von absolutem Alkohol versetzt. Der Niederschlag wurde wieder in heißem Wasser aufgenommen. Er löste sich nur teilweise. Das Filtrat wurde nochmals mit Alkohol niedergeschlagen, dieser Niederschlag bei 105° unter vermindertem Druck über Phosphorpentoxyd getrocknet und analysiert.

<sup>1)</sup> Als Farbenreaktion wurde die Orcinprobe benutzt. Der eine von uns (Levene), in Gemeinschaft mit C. L. Alsberg, hat vor einigen Jahren die Beobachtung gemacht, daß, in Anwesenheit von nur einer Spur von Kupfer, Orcin mit allen Kohlehydraten den typischen Farbstoff bildet.

<sup>2)</sup> Hoppe-Seylers Ztschr. für physiol. Chem. 45, 370 [1905].

0.1741 g Sbst.: 0.1592 g CO<sub>2</sub>, 0.0460 g H<sub>2</sub>O. — 0.2026 g Sbst. (Kjeldahl): 8.70 ccm Schwefelsäure (1 ccm = 0.00113 g Stickstoff). — 0.5520 g Sbst.: 0.1020 g Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>. — 0.1290 g Sbst.: 0.0460 g BaSO<sub>4</sub>.

Für den Komplex thyminglucophosphorsäures Barium läßt sich Folgendes berechnen:

C<sub>11</sub>H<sub>13</sub>N<sub>2</sub>P O<sub>10</sub>Ba. Ber. C 26.18, H 2.98, N 5.54, P 6.04, Ba 27.23.  
Gef. » 24.89, » 3.00, » 5.97, » 5.12, » 29.19.

Es wurde dann versucht, diese Substanz weiter zu reinigen. Zu diesem Zwecke wurde sie wieder in kochendem Wasser aufgenommen. Auch dieses Mal bildete sich ein in Wasser löslicher und ein unlöslicher Teil. Der lösliche Teil, mit Alkohol niedergeschlagen, enthielt nur 4.65 % Phosphor, der unlösliche gab bei der Analyse die folgenden Zahlen:

0.2000 g Sbst. (Kjeldahl): 8.1 ccm Schwefelsäure (1 ccm = 0.00113 g Stickstoff). — 0.5365 g Sbst.: 0.1227 g Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>. — 0.2674 g Sbst.: 0.1217 g BaSO<sub>4</sub>.

C<sub>11</sub>H<sub>13</sub>N<sub>2</sub>P O<sub>10</sub>Ba. Ber. N 5.54, P 6.06, Ba 27.23.  
Gef. » 5.65, » 6.36, » 26.89.

Bei weiterer Spaltung der Substanz erhielt man Phosphorsäure, Thymin und Lävulinsäure. Die Spaltung wurde im Autoklaven bei 175° durch 4-stündiges Erhitzen mit 25-proz. Schwefelsäure ausgeführt. Das Rohprodukt wurde zum Versuch gebraucht. Die resultierende, braune Flüssigkeit wurde mit Äther extrahiert. Der ätherische Auszug wurde an der Luft verdunsten gelassen und aus dem Rückstande das Silbersalz der Lävulinsäure dargestellt.

0.1407 g Sbst. (nicht umkrystallisiert) gaben 0.0685 g Silber.

C<sub>5</sub>H<sub>7</sub>O<sub>3</sub>Ag. Ber. Ag 48.43. Gef. Ag 48.69.

Die Flüssigkeit wurde nach dem Extrahieren mit Äther vom Überschuß von Schwefelsäure und Phosphorsäure mittels Barytwasser befreit und eingedampft. Es krystallisierte dabei Thymin in typischer Form aus.

0.1315 g Sbst : 25.25 ccm N (14.0°, 759 mm).

C<sub>8</sub>H<sub>8</sub>N<sub>2</sub>O<sub>2</sub>. Ber. N 22.22. Gef. N 22.55.

Daß die Entstehung der Substanz nicht auf einem Zufalle beruhte, ist daraus ersichtlich, daß die Substanz, die Schmiedeberg und Alsberg auf anderem Wege erhalten hatten und als Heminucleinsäure betrachteten, mit unserer Substanz fast identisch war; es geht dies auch aus dem Vergleich der Zahlen für die bariumfreie Thymoglucophosphorsäure mit den von Alsberg für die Heminucleinsäure gefundenen Zahlen hervor.

$C_{11}H_{17}N_2PO_{10}$ . Ber. C 35.87, H 4.62, N 7.60, P 8.47.

Gef. » 36.30, » 5.98, » 8.23, » 8.14. (Alsberg)<sup>1)</sup>.

Man darf also diese Substanz als einen Bestandteil der Nucleinsäure annehmen und als ein Mononucleotid betrachten.

<sup>1)</sup> Arch. für exp. Path. u. Pharmacol. **51**, 240 [1904].



**319. P. A. Levene und W. A. Beatty:**  
**Über das Vorkommen von Prolinglycyanhydrid bei der**  
**tryptischen Verdauung der Gelatine.**

[Aus dem Rockefeller-Institute for Medical Research, New York.]

(Eingegangen am 7. Mai 1906; mitget. in der Sitzung von Hrn. O. Diels.)

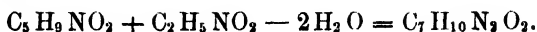
Bei der tryptischen Verdauung von Gelatine ist es Levene<sup>1)</sup> gelungen, eine Substanz von der Zusammensetzung  $C_7H_{10}N_2O_2$  zu gewinnen. Sie krystallisierte in prismatischen Nadeln, besaß einen Schmelzpunkt von 182–183°, bildete mit Kupferoxyd kein Salz und mit Platinchlorid keine Verbindung. — Die Substanz schmeckte bitter und entwickelte bei der Sublimation Pyrrol. Ihre Darstellungsweise ist von Levene und Wallace<sup>2)</sup> angegeben.

Es wurde nun versucht, die Substanz weiter zu spalten, und es gelang dabei,  $\alpha$ -Prolin und Glykokoll als Bestandteile zu erkennen.

<sup>1)</sup> Journ. of Exper. Med. Vol. VIII, 180 [1906].

<sup>2)</sup> Ztschr. für physiol. Chem. 47, 148 [1906].

Man ist also berechtigt, die Substanz als Prolin-glycyl-anhydrid zu betrachten.



1.5 g der Substanz wurden im zugeschmolzenem Rohr 5 Stunden mit konzentrierter Salzsäure auf 150° erhitzt. Das Reaktionsprodukt wurde von überschüssiger Salzsäure durch Destillation unter vermindertem Druck und endlich mit Silbersulfat befreit. Das Silber und die Schwefelsäure wurden auf übliche Weise entfernt und die Spaltungsprodukte zur Trockne verdampft. Der Rückstand schmeckte süß. Er wurde mittels heißen Methylalkohols so lange extrahiert, bis der Rückstand keine Pyrrol gebende Substanzen mehr enthielt. Der unlösliche Teil besaß krystallinische Beschaffenheit und bildete ein rotes Sublimat; mit alkoholischer Pikrinsäure gab er ein krystallinisches Pikrat. Er besaß also alle Eigenschaften des Glykokolls.

0.0881 g Sbst.: 14.4 ccm N (über 50-proz. KOH) (18.5°, 747 mm).

$\text{C}_2\text{H}_5\text{NO}_2$ . Ber. N 18.66. Gef. N 18.88.

Der methyllalkoholische Auszug wurde eingedampft und der Rückstand mit absolutem Alkohol extrahiert. Dieser Auszug wurde auf ein ganz kleines Volumen konzentriert; es bildete sich dann beim Stehen ein krystallinischer Niederschlag, der alle Eigenschaften des aktiven  $\alpha$ -Prolins besaß. Bei der Verbrennung dieses Präparates ging die Wasserbestimmung durch Unfall verloren, der Kohlenstoffgehalt betrug 52.28%. Es wurde deswegen eine zweite Spaltung unternommen. • Bei diesem Experimente kam 1.0 g der Substanz zum Verbrauch. Die Spaltung wurde bei 125° ausgeführt. In allen anderen Hinsichten wurde dasselbe Verfahren wie bei dem ersten Experiment befolgt. Das Reaktionsprodukt wurde aber statt mit Methylalkohol mit absolutem Äthylalkohol extrahiert; aus diesem krystallisierte (nach Eindunsten)  $\alpha$ -Prolin aus. Im Xylolbad getrocknet, hatte die Substanz einen Schmelzpunkt von 205° und die folgende Zusammensetzung:

0.1037 g Sbst.: 0.1995 g  $\text{CO}_2$ , 0.0740 g  $\text{H}_2\text{O}$ .

$\text{C}_5\text{H}_9\text{NO}_2$ . Ber. C 52.17, H 7.82.

Gef. » 52.46, » 7.92.

Die Ausbeute an Glykokoll im zweiten Experimente betrug 0.370 g, an Prolin 0.400 g.

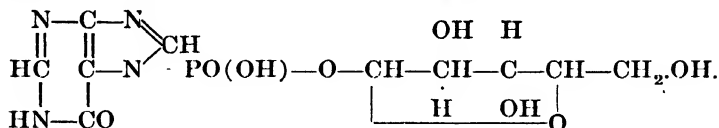
# PART I.

## 456. P. A. Levene und W. A. Jacobs: Über die Inosinsäure.

[Aus dem Rockefeller Institute for Medical Research, New York.]  
(Eingegangen am 13. Juli 1908; yorgetr. in der Sitzung von Hrn. C. Neuberg.)

Die Inosinsäure, welche wegen des Vorhandenseins einer mit Purinbase und Kohlehydrat gespaarten Phosphorsäure im Moleküle als eine einfache Nucleinsäure angesehen werden darf, ist von Liebig<sup>1)</sup> im Fleischextrakt entdeckt worden. Ihre wirkliche Natur ist zuerst von Haiser<sup>2)</sup> eingehend untersucht worden, ohne dass er aber ihre Struktur endgültig aufklären konnte. — Im vorigen Jahre begannen Neuberg und Brahm<sup>3)</sup> und auch gleichzeitig Bauer<sup>4)</sup> die Säure zu untersuchen; hauptsächlich lag ihnen daran, die Natur des im Molekül vorliegenden Kohlehydrates aufzuklären.

Nach der Annahme von Neuberg und Brahm kommt der Substanz die folgende Zusammensetzung zu:



Die Zuckerkomponente wurde also als *l*-Xylose angesehen. — Nach Bauer soll die Zusammensetzung die folgende sein:  $(\text{HO})_2\text{PO.O}.\text{CH}_2[\text{CH.OH}]_3.\text{CH}:(\text{C}_5\text{H}_3\text{N}_4\text{O})$ , als Pentose eine *d*, *l*-Arabinose vorliegen.

Neuberg und Brahm und auch Bauer nehmen die Existenz einer Pentose-Phosphorsäure an, ohne sie wirklich isoliert zu haben. — Haiser aber hatte die Substanz in Händen, ohne ihre wirkliche Natur aufgeklärt zu haben.

Als die Arbeit von Neuberg und Brahm erschien, war schon einer von uns mit der Darstellung grösserer Quantitäten von Inosinsäure beschäftigt. — Die Resultate der letzten beiden Untersuchungen haben uns überzeugt, dass man zu bestimmteren Resul-

<sup>1)</sup> Ann. d. Chem. **62**, 317 [1847].    <sup>2)</sup> Monatsh. für Chem. **16**, 190 [1895].

<sup>3)</sup> Biochem. Ztschr. **5**, 438 [1901].

<sup>4)</sup> Hofmeisters Beiträge **10**, 345 [1907].

taten nur dann gelangen würde, wenn man grössere Mengen vom Ausgangsmaterial zur Verfügung hätte. Deswegen wurde die Arbeit fortgesetzt.—23 kg Liebig's Fleischextrakt wurden genau nach der Methode von H a i s e r verarbeitet; wir kamen dadurch in den Besitz von mehr als 120 g des umkrystallisierten Bariumsalzes. — Im Durchschnitt erhielten wir 5—6 g pro kg. Wir wollen dabei bemerken, dass der Gebrauch von absolutem Alkohol für die Extraktion kaum nötig ist. Die Ausbeute war grade so gut beim Extrahieren des Fleischextraktes mittels 95 prozent. Alkohols. Die Untersuchung wurde auf die Natur der Pentose und auf die Bindungsart der einzelnen Komponenten gerichtet. Die Resultate unserer Untersuchungen lassen die Annahme berechtigt erscheinen, dass das Kohlehydrat mit der Phosphorsäure eine G l y k o p h o s p h o r s ä u r e bildet, und dass die Base an die Aldehydgruppe des Zuckers gebunden ist, und zwar aus den folgenden Gründen:

Wird Inosinsäure mittels verdünnter Schwefelsäure hydrolysiert, so gelangt man zu einem Punkte, bei welchem die ursprüngliche Linksdrehung sich in Rechtsdrehung umwandelt; dann bleibt die Drehung längere Zeit konstant. An diesem Punkt der Hydrolyse gelang es uns, ein Kupfersalz und zwei Bariumsalze, eines in schön krystallinischer Form, einer gespaarten Phosphorsäure zu gewinnen. Die Säure besass stark reduzierende Eigenschaften für F e h l i n g s c h e Lösung. Die Substanz ist beim Kochen mittels Säuren ziemlich resistent. Im Gegensatz dazu änderte sich das ursprüngliche Drehungsvermögen der Lösung bei langdauerndem Erhitzen mit Alkalien nicht wesentlich. Die Lösung enthält dabei keine freie Base und kein freies Kohlenhydrat, wohl aber freie Phosphorsäure, und es gelingt so, einen Komplex zu erhalten, welcher scheinbar aus Kohlehydrat und Hypoxanthin besteht.

Über die Natur des Fünfkohlenstoff-Zuckers sind wir bisher zu einer bestimmten Ansicht nicht gelangt. Die analytischen Zahlen, welche Liebig und H a i s e r angeben, stimmen am besten für eine Pentose, auch der Wasserstoffgehalt der von uns dargestellten gepaarten Phosphorsäure scheint diese Ansicht zu bestätigen. Es erwies sich aber als ganz unmöglich, Beweise für das Vorliegen einer Arabinose oder einer Xylose beizubringen. Bei der Destillation der Inosinsäure, wie auch bei der des zweibasischen Bariumsalzes der gepaarten Phosphorsäure waren die Ausbeuten an Furfurol zu gering im Vergleich mit denen, die man bei Anwesenheit einer Arabinose oder einer Xylose erwarten durfte. Alle Bemühungen, ein Phenylsazon mit den Eigenschaften eines Xylosazones oder eines Arabinosazones zu erhalten, waren vergeblich, obwohl die Spaltung solange fortgesetzt wurde, dass die ursprünglich links-

drehende Lösung eine konstante Rechtsdrehung annahm. Bei der Oxydation mittels Brom und gleichzeitiger Spaltung mittels Bromwasserstoffsäure erhielten wir keine Xylonsäure, auch keine Arbonsäure, sondern ein Produkt, welches noch gebundene Phosphorsäure enthielt und nicht mehr/reduzierte. Auf Grund dieser Beobachtungen wären wir bereit, die Anwesenheit einer Tetrose-carbonsäure anzunehmen, wenn der Wassertoffgehalt der Inosinsäure dieser Ansicht nicht widerspräche. Weitere Versuche über die Oxydation der Inosinsäure sind jetzt im Gange, sie sollen auch diese Frage aufklären.

Es wurde auch daran gedacht, dass im Moleküle der Inosinsäure vielleicht noch eine Hexose vorläge. Es gelang uns aber nicht, aus 5 g des Bariumsalzes nach der üblichen Behandlung Lävulinsäure nachzuweisen.

### Experimenteller Teil.

10.0 g inosinsaures Barium wurden vom Barium befreit und mit 200 ccm 5-proz. Schwefelsäure bei 50° im Ostwaldschen Thermostaten 96 Stunden erhitzt. Die ursprüngliche Drehung war  $-0.5^\circ$  (im 1-dm-Rohr). Nach 96 Stunden erreichte die Drehung  $+0.33^\circ$  und blieb dann konstant.

Das Experiment wurde dann unterbrochen. Das Hypoxanthin wurde mittels Silbersulfat entfernt, das Filtrat vom Silber mittels Schwefelwasserstoff befreit und die Schwefelsäure und Phosphorsäure mittels Bariumcarbonat entfernt. Die resultierende Lösung enthielt die Bariumsalze der unzersetzt gebliebenen Inosinsäure und der Kohlehydrat-phosphorsäure. Um diese zu trennen, haben wir die Lösung bis zu einem ganz kleinen Volumen bei 35° eingedampft, und bei 0° 24 Stunden lang stehen gelassen. Das Bariumsalz der Inosinsäure krystallisierte dabei aus. Das Filtrat wurde weiter eingedampft und wieder bei 0° stehen gelassen. Der sich hierbei bildende kleinere Niederschlag wurde abfiltriert und das Filtrat mit dem gleichen Volumen Alkohol behandelt. Es bildete sich dabei ein klebriger Niederschlag, welcher beim Stehen bei 0° eine harte, spröde Konsistenz annahm. Der Niederschlag wurde dann abfiltriert, mit Alkohol und Äther gewaschen und im Vakuum-Exsiccator über Schwefelsäure bis zum konstanten Gewicht getrocknet. Seine Menge betrug dann 2.5 g. Dieser Niederschlag enthielt kaum Spuren von Stickstoff und Phosphorsäure und reduzierte Fehlingsche Lösung beim Erhitzen. Das Salz wurde mit einer kleinen Menge Wasser ausgekocht: der grössere Teil ging in Lösung über, beim Stehen im Eisschrank bildete sich kein Niederschlag mehr. Die Lösung wurde dann mit dem gleichen Volumen Alkohol behandelt, es bildete sich wieder ein amorpher Niederschlag, welcher nach längerem Stehen harte Konsistenz annahm. Er wurde dann abfiltriert und mit wenig heissem Wasser behandelt; der unlösliche Teil abfiltriert, mit Alkohol und Äther gewaschen und zur Analyse gebracht.

Die Substanz enthielt keinen Stickstoff, keine freie Phosphorsäure und reduzierte Fehlingsche Lösung beim Erhitzen.

0.1026 g der Substanz bei 100° im Vakuum über Phosphorpentoxyd getrocknet: 0.0700 g  $\text{Ba}_3(\text{PO}_4)_2$ .

$\text{Ba}_3(\text{C}_6\text{H}_4\text{O}_6\text{P})_2$ . Ber. Ba 69.48. Gef. Ba 68.22.

0.1782 g Bariumsalz, in 1.2 ccm *n*-HCl (1 Mol.) und 3 ccm  $\text{H}_2\text{O}$  gelöst, drehten in  $\frac{1}{2}$ -dm-Rohr bei Na-Licht 0.08° nach rechts. Gesamtgewicht der Lösung 4.9009 g, mithin  $[\alpha]_D^{20} = 4.4^\circ$ .

Aus der Mutterlauge wurde durch fraktionierte Fällung mittels Alkohol noch eine Bariumverbindung einer gepartten Phosphorsäure erhalten, welche dieselbe Zusammensetzung besass.

### Experiment 2.

10.0 g des Bariumsalzes wurden von Barium mittels Schwefelsäure befreit und zur Trockne eingedampft. Der Rückstand wurde in 200 ccm 5-prozentiger Schwefelsäure aufgelöst. Die Lösung wurde nur 3 Tage auf 50° erhitzt. Das Hypoxanthin wurde dann mittels Silbersulfat entfernt und das Filtrat mit Schwefelwasserstoff behandelt. Das Filtrat wurde dann mit Bariumcarbonat 10 Minuten gekocht. Das Filtrat wurde auf ein kleineres Volumen eingedampft und im Eisschrank der Krystallisation überlassen. Nach 48 Stunden wurde das Bariumsalz der noch unveränderten Inosinsäure beinahe vollständig ausgeschieden. Das Filtrat, dessen Menge ungefähr 5 ccm betrug, wurde im Eisschrank bei 0° stehen gelassen. Nach einigen Wochen begannen sich dicke, grobe Krystallbüschel auszuschcheiden, und nach mehreren Tagen war die Krystallisation vollendet. Die Krystalle wurden abgesaugt, mit Wasser gewaschen und an der Luft getrocknet. Die Ausbeute betrug 1.5 g. —

Das Salz war frei von freier Phosphorsäure, aber nach dem Zersetzen mittels Salpetersäure zeigte es eine starke Phosphorsäurereaktion. Es reduzierte Fehling'sche Lösung stark, und gab mittels Orein und Salzsäure die charakteristische Pentosereaktion und enthielt keinen Stickstoff. Das Salz war in kaltem Wasser schwer löslich; beim Erhitzen erlitt es eine teilweise Zersetzung unter gleichzeitiger Ausscheidung eines basischen Bariumsalzes, welches aller Wahrscheinlichkeit nach mit dem im vorigen Experimente erhaltenen Bariumsalze identisch ist. Deshalb wurde das Salz nicht umkrystallisiert und zur Analyse gebracht. Das Salz enthielt Krystallwasser. Das lufttrockne Präparat wurde im Vakuum über Phosphorpentoxyd bei 78° bis zum konstanten Gewicht erhitzt.

0.1797 g der Substanz verloren 0.0353 g Wasser.

$\text{C}_6\text{H}_4\text{O}_6\text{PBa} + 5 \text{H}_2\text{O}$ . Ber. 19.19.

$\text{C}_6\text{H}_4\text{O}_6\text{PBa} + 5 \text{H}_2\text{O}$ . Ber. 19.78.

Gef. 19.65.

0.1701 g lufttrockenes Präparat gaben 0.0798 g  $\text{CO}_2$ , 0.0648 g  $\text{H}_2\text{O}$ . —  
0.1994 g lufttrockene Substanz gaben 0.0988 g  $\text{BaSO}_4$ .

$\text{C}_6\text{H}_4\text{O}_6\text{PBa} + 5\text{H}_2\text{O}$ . Ber. C 12.60, H 3.62, Ba 29.29.

$\text{C}_6\text{H}_4\text{O}_6\text{PBa} + 5\text{H}_2\text{O}$ . Ber. " 13.19, " 4.17, " 30.20.

Gef. " 12.81, " 4.23, " 29.13.

Um zu sehen, ob das Furfurol, welches durch die Destillation mittels Salzsäure erhalten wurde, einer Pentose entspricht, haben wir eine Furfurolbestimmung gemacht. Die Substanz wurde mittels Salzsäure (spez. Gew. 1.6) destilliert, bis ungefähr 500 ccm übergegangen waren und kein Furfurol mehr im Destillat zu bemerken war.

So wurden aus 0.1994 g des Salzes 0.0184 g des Phloroglucides erhalten, also ungefähr  $\frac{1}{3}$  von der erwarteten Menge, wenn das Kohlehydrat eine Pentose wäre. In gleicher Weise haben wir das Bariumsalz der Inosinsäure selbst behandelt und zwar mit ähnlichem Erfolge. Es wurden aus 0.5207 g des Bariumsalzes nur 0.0313 g Phloroglucid erhalten, während eine Pentose im Moleküle ungefähr 0.12 g Phloroglucid erfordert. Dieses Resultat steht innerhalb der Versuchsfehler gerade im richtigen Verhältnis mit der Zahl aus der Kohlehydrat-Phosphorsäure.

Dieses Resultat steht allerdings im Widerspruch mit der Angabe von Neuberger und Brahm, die Furfurol, entsprechend einer Xylose, erhalten haben wollen, und mit denen von Bauer, welcher eine Ausbeute an Furfurol, einer Arabinose entsprechend, fand. Nach unserer Erfahrung sind wir nicht berechtigt, eine Xylose oder eine Arabinose im Moleküle anzunehmen.

### Experiment 3. Alkalische Hydrolyse.

5.0 g des Bariumsalzes wurden vom Barium befreit und mit einem Mol.-Gew. Natriumhydroxyd behandelt. Die Lösung wurde im Vakuum zur Trockne eingedampft und der Rückstand in 100 ccm 5-prozentiger Kalilauge aufgelöst und im Ölbade auf 130–140° erhitzt. Das ursprüngliche Drehungsvermögen der Substanz betrug  $-2.05^\circ$  (im 1.9-dm-Rohr). Nach 14-stündigem Erhitzen änderte sich das Drehungsvermögen nicht mehr wesentlich. Die Lösung reduzierte Fehling'sche Lösung nicht; mittels ammoniakalischer Silberlösung gelang es nicht, einen Niederschlag zu erhalten. In 20 ccm der ursprünglichen Lösung wurde eine Phosphorsäurebestimmung ausgeführt. Die Lösung wurde mit kalter Salpetersäure angesäuert und mit Ammoniummolybdatlösung behandelt. Ein Erhitzen der Lösung wurde vermieden. Aus dem Molybdänniederschlag gelang es, 0.0553 g  $\text{Mg}_2\text{P}_2\text{O}_7$  zu gewinnen. Dabei wurden also 30% der ursprünglichen Phosphorsäure abgespalten.

## PART II.

### 45. P. A. Levene und W. A. Jacobs: Über Inosinsäure.

[Zweite Mitteilung.]

[Aus dem Rockefeller Institute for Medical Research, New York, N. Y.]  
(Eingegangen am 21. Dezember 1908.)

In einer früheren Mitteilung<sup>1)</sup> haben wir gezeigt, dass man bei der Hydrolyse der Inosinsäure in saurer Lösung zu einem Zustande gelangen kann, in welchem die ursprünglich linksdrehende Lösung eine konstante Rechtsdrehung annimmt. Aus dieser Lösung konnten wir ein krystallinisches Bariumsalz einer Pentose-Phosphorsäure isolieren, welches Fehlingsche Lösung stark reduzierte. Wir hatten weiter ein Experiment erwähnt, in welchem wir die Säure der alkalischen Hydrolyse unterwarfen. Hierbei wurde Phosphorsäure abgespalten, ohne dass dadurch in der Lösung reduzierende Eigenschaften oder freies Hypoxanthin entstand. Aus dieser Lösung konnten wir eine sehr kleine Menge einer Silberverbindung erhalten, welche mittels Orcin und Salzsäure die charakteristische Pentose-Reaktion zeigte, auch die Anwesenheit von Hypoxanthin konnten wir im Moleküle nachweisen, aber keinen Phosphor. Dieser Komplex bestand scheinbar aus Hypoxanthin und einem Zucker, er zeigte keine reduzierenden Eigenschaften. Wir waren deshalb zu der Ansicht gelangt, dass im Inosinsäure-Moleküle das Purin glykosidartig an Zucker gebunden ist, und dass die Phosphorsäure esterartig an einem Hydroxyl des Zuckers steht. Es ist uns nun jetzt gelungen, weitere Beweise für die Richtigkeit unserer Ansicht über die Struktur der Substanz zu gewinnen. Beim Erhitzen einer wässrigen Lösung des Bariumsalzes im geschlossenen Rohr auf 125–130° wird die Phosphorsäure als Bariumphosphat abgespalten, während die resultierende Flüssigkeit keinen Pentose- oder Hypoxanthingehalt zeigte. Aus dieser Lösung wurde ein Körper erhalten, welcher sich als ein Zucker-Hypoxanthin-Komplex erwies. Dieser Körper sollte identisch sein mit einer Substanz, welche Haiser und Wenzel<sup>2)</sup> vor kurzem aus dem Carnin isoliert haben, und die sie Inosin nannten. Zum Zwecke der Vergleichung der beiden Substanzen haben wir Inosin genau nach der

<sup>1)</sup> Diese Berichte **41**, 2703 [1908].

<sup>2)</sup> Monatsh. f. Chem. **39**, 157 [1908].

Vorschrift von H a i s e r dargestellt und mit der aus der Inosinsäure erhaltenen Substanz verglichen. Sie erwiesen sich hierbei als identisch. In einem anderen Experiment wurde das Erhitzen des Bariumsaltzes länger fortgesetzt, dabei konnten wir aus dem Reaktionsgemisch Carnin selbst isolieren. H a i s e r und W e n z e l selbst haben die Ansicht geäußert, dass wahrscheinlich die Inosinsäure und das Carnin von derselben Muttersubstanz herkommen. Wir glauben uns jetzt zu der Annahme berechtigt, dass das Carnin ein Spaltungsprodukt der Inosinsäure ist. Wir sind auch mit der Frage über die Natur der Pentose beschäftigt, und wir hoffen, bald die Resultate dieser Untersuchungen veröffentlichen zu können.

5.0 g des Bariumsaltzes wurden mit 200 ccm Wasser im Einschliessrohr aufgelöst. Das Rohr setzten wir vor dem Erkalten in ein Ölbad von 100° ein, um eine Ausscheidung des Salzes zu vermeiden. Das Bad wurde dann auf 125–130° 6 Stunden lang gehalten. Nach dem Erkalten wurde vom Niederschlag abfiltriert, welcher aus Bariumphosphat, inosinsaurem Barium und basischem inosinsaurem Barium bestand. Die Lösung, welche schwach saure Reaktion zeigte, wurde dann auf 50 ccm eingedampft und im Eischrank der Krystallisation überlassen. Nach 24 Stunden wurde vom ausgeschiedenen inosinauren Barium abfiltriert. Das Filtrat zeigte keine reduzierenden Eigenschaften, auch enthielt es kein freies Purin. Da die Lösung noch beträchtliche Mengen von freier Inosinsäure enthielt, konnten wir kein Inosin durch Eindampfen erhalten. Deshalb haben wir nach dem Verfahren von H a i s e r und W e n z e l durch fraktionierte Fällung mittels Blei unser Ziel erreicht. Die Lösung wurde mit Ammoniak neutral gemacht und mit Bleiessig versetzt, bis sich kein Niederschlag mehr bildete. Der Niederschlag, welcher aus Bleiverbindungen der Inosinsäure und der Phosphorsäure bestand, wurde abfiltriert. Das Filtrat wurde mit Bleiessig und Ammoniak abwechselnd behandelt, bis die Fällung vollendet war. Der Niederschlag wurde abgesaugt, gründlich ausgewaschen und mittels Schwefelwasserstoff zersetzt. Das Filtrat war durch Ammoniumsulfid verunreinigt, es wurde zur Reinigung hiervon mit Essigsäure schwach sauer gemacht und unter vermindertem Druck eingedampft. Der Rückstand wurde in Wasser gelöst und die Lösung mit Baryt schwach alkalisch gemacht und wieder bei vermindertem Druck zur Trockne eingedampft. Der Rückstand wurde nochmals in Wasser gelöst und das Barium quantitativ mittels Schwefelsäure entfernt. Das Filtrat wurde nach dem Behandeln mit Tierkohle bei vermindertem Druck auf ein kleines Volumen eingedampft, worauf sich der Purin-Pentose-Komplex in rein weissen, perlmutterartig glänzenden Nadeln ausschied. Die Krystalle wurden abgesaugt, mit wenig kaltem Wasser

nachgewaschen und an der Luft bis zum konstanten Gewicht trocknen gelassen. Unter dem Mikroskop bestand der Körper aus langen, rechtwinkligen Tafeln; gerade dasselbe Aussehen zeigte eine Probe Inosin, welches nach der Methode Haisers aus Carnin über die Acetylverbindung dargestellt war. Von den Angaben Haisers wich es aber im Schmelzpunkt ab. Das Inosin aus Inosinsäure sowohl, als aus Carnin (an der Luft getrocknet) fing, im Capillarrohr erhitzt, schon bei  $85^{\circ}$  (korr.) an, zu sintern, und gegen  $89-90^{\circ}$  schmolz es zu einer blasenhaltigen Flüssigkeit; bei weiterem Erhitzen blähte es sich auf. Der Unterschied dieses Befundes von der Angabe Haisers beruht darin, dass das lufttrockne Präparat bei der Analyse Krystallwassergehalt anzeigte. Nach dem Trocknen im Vakuum über Schwefelsäure bei  $18^{\circ}$  verhielt sich unsere Substanz gerade wie die von Haiser und Wenzel. Sie fing gegen  $215^{\circ}$  an, zu verkohlen.

In allen anderen Eigenschaften waren beide Verbindungen identisch; zur Analyse wurde das lufttrockne Präparat benutzt.

0.1551 g Subst. verloren, im Vakuum über  $P_2O_5$  bei  $78^{\circ}$  erhitzt: 0.0182 g  $H_2O$ .

$C_{10}H_{12}N_4O_5 + 2H_2O$ . Ber.  $H_2O$  11.83. Gef.  $H_2O$  11.73.

0.1369 g wasserfreie Subst.: 0.2216 g  $CO_2$ , 0.0568 g  $H_2O$ .

$C_{10}H_{12}N_4O_5$ . Ber. C 44.77, H 4.47.  
Gef. " 44.14, " 4.61.

Durch diesen Befund ist die Konstitution der Inosinsäure, was die Anordnung ihrer Komponenten betrifft, vollständig aufgeklärt.

## 185. P. A. Levene und W. A. Jacobs: Über Inosinsäure.

[Dritte Mitteilung.]

[Aus dem Rockefeller Institute for Medical Research.]

(Eingegangen am 5. März 1909.)

Über die Natur der Kohlehydratgruppe der Inosinsäure liegen die Ansichten von Neuberg und Brahn<sup>1)</sup> und die von Bauer<sup>2)</sup> vor. Die ersten Forscher gelangten zu der Ansicht, daß sie *l*-Xylose in den Händen hatten, während Bauer glaubte, Beweise für das Vorliegen der *d, l*-Arabinose geliefert zu haben. Neuberg und Brahn gründeten ihre Ansicht auf die folgenden Befunde. Bei der Destillation der Inosinsäure mit Salzsäure vom spez. Gewicht 1.06 erhielten sie eine Menge von Furfurol-Phloroglucid, welche man nach der Theorie für eine Xylose erwarten sollte. Zweitens, das von ihnen erhaltene Osazon hatte den Schmelzpunkt und das Drehungsvermögen des Phenyl-Xylosazons. Im Gegensatz dazu entsprach die Ausbeute an Phloroglucid im Experiment von Bauer der für *d, l*-Arabinose verlangten. Im negativen Drehungsvermögen seines Osazons sah er einen weiteren Beweis für die Richtigkeit seiner Ansicht.

Nun haben wir in unserer ersten Mitteilung<sup>3)</sup> behauptet, daß unsere Befunde beiden Ansichten widersprächen, obwohl wir keine positive Ansicht über die exakte Natur der Substanz hatten. Wir behaupteten, daß es uns nicht gelungen war, die nach der Theorie für die Xylose oder die Arabinose verlangte Ausbeute an Phloroglucid zu erhalten, und daß das Phenylsazon nicht die Eigenschaften des Phenylxylosazons oder *dl*-Arabinosazons besaß. Das Vorhandensein einer Tetrose-Carbonsäure haben wir ausgeschlossen. Unsere Bemerkungen waren von Hrn. Neuberg und Brahn stark angegriffen worden. Nun ist es uns jetzt gelungen, den Zucker in schön krystallinischer Form zu erhalten und auf diesem Wege für die Richtigkeit unserer ersten Behauptung weitere Beweise zu liefern. Der krystallinische Zucker unterscheidet sich von der Xylose und der Arabinose im Schmelzpunkt im Drehungsvermögen, in dem Drehungsvermögen des Phenylsazons, im Schmelzpunkt des Benzyl-Phenylhydrazons. Weiter gelang es nicht, aus dem krystallinischen Zucker das Cadmiumsalz der Xylonsäure zu erhalten. Diesen Zucker haben wir zwar nicht direkt aus der Inosinsäure, wohl aber aus dem Inosin, welches

<sup>1)</sup> Biochem. Ztschr. 5, 438 [1901]; diese Berichte 41, 3376, [1908].

<sup>2)</sup> Hofmeisters Beiträge 10, 345 [1907].

<sup>3)</sup> Diese Berichte 41, 2703 [1908].

nach Haiser<sup>1)</sup> und Wenzel aus Carnin dargestellt worden war, erhalten. Aus dem Inosin ist es schon Haiser und Wenzel gelungen, durch saure Hydrolyse einen süß schmeckenden Sirup zu erhalten, woraus sie ein Osazon darstellten, welches genau denselben Schmelzpunkt besaß, welchen wir beobachteten. Den Zucker aber konnten sie nicht krystallinisch erhalten, und außer dem Schmelzpunkt haben sie keine weiteren Eigenschaften erwähnt.

Über die Identität des Inosins aus Carnin und aus Inosinsäure haben wir in der zweiten Mitteilung<sup>2)</sup> berichtet, wir dürfen aber noch hinzufügen, daß auch in ihrer optischen Aktivität die beiden Substanzen identisch sind, ebenso auch darin, daß die Phenylosazone der Zuckergruppen der beiden Substanzen einen ähnlichen Schmelzpunkt und dasselbe Drehungsvermögen besaßen. Es liegt also kein Zweifel vor, daß die Zucker der Inosinsäure und des Inosins identisch sind.

Was die Ausbeute an Phloroglucid betrifft, so ist es uns auch jetzt nicht gelungen, die Werte von Neuberg und Brahn oder von Bauer zu erhalten, obwohl wir alle Maßregeln genau verfolgten, wohl aber gelingt es, bei der Destillation des Inosins eine Ausbeute an Phloroglucid zu erhalten, die dem für eine Pentose berechnendem entspricht.

Nach allem ist man nun berechtigt anzunehmen, daß die Zuckergruppe der Inosinsäure zu den Pentosen gehört, daß sie aber in ihren Eigenschaften von *l*-Xylose und *d,l*-Arabinose sich unterscheidet. Weitere Versuche über die Natur der Substanz sind jetzt im Gange.

## Experimenteller Teil.

### Darstellung des Pentosazones aus der Inosinsäure.

Bei der Darstellung eines reinen Pentosazones aus Inosinsäure sind wir auf viele Schwierigkeiten gestoßen. Durch saure Hydrolyse oder durch Kochen der freien Inosinsäure in wäßriger Lösung wird, wie bekannt, intermediär Pentose-Phosphorsäure gebildet. In Anwesenheit dieser Substanz gelingt es nur unter großen Schwierigkeiten ein reines Pentosazon zu erhalten. Es bilden sich gewöhnlich harzartige, phosphorhaltige Substanzen, welche man nur mit großer Mühe in Krystallform überführen kann, — aber es gelang uns nie, sie ganz rein zu erhalten. Auch mittels Alkohol-Extraktion gelang es nicht, die Pentose vollkommen von der phosphorhaltigen Substanz zu trennen. In diesem Versuche wurde die zur Hydrolyse benutzte Schwefelsäure mittels Bariumcarbonat entfernt, dann wurde das Hypoxanthin zum Ausscheiden sich überlassen. Die Mutterlauge von diesem wurde unter vermindertem Druck eingedampft, und aus dem Rückstand versuchte man die Pentose mit Alkohol

<sup>1)</sup> Monatsh. für Chem. 29, 157 [1908].

<sup>2)</sup> Diese Berichte 42, 335 [1909].

auszulaugen. Die Auszüge enthielten noch immer phosphorhaltige Körper, und die Darstellung eines reinen Phenylpentosazonones gelang nicht. Wir haben einmal aus 10 g des Bariumsalzes nach 6-stündigem Kochen mittels 2proz. Schwefelsäure die gepaarte Phosphorsäure vollständig aufgespalten und ein Pentosazon dargestellt. Die Ausbeute betrug nur 0.050 g, und die Lösung der Substanz war zu dunkel, um eine optische Untersuchung zu erlauben. Wir haben aber unser Ziel auf anderem Wege erreicht.

2.0 g Barium-Inosinat wurden in 150 ccm heißen Wassers aufgelöst, mit ein Mol normaler Schwefelsäure versetzt und 6 Stunden am Rückflußkühler gekocht. — Die Lösung wurde dann mit wenig Bariumcarbonat gekocht und das Filtrat unter vermindertem Druck auf ein kleines Volumen, eingedampft. Nach dem Stehen im Eisschrank wurde von dem ausgeschiedenen Hypoxanthin abfiltriert und das Filtrat mit Kupferacetat im Überschuss versetzt. Hierbei entstand sofort ein grüner Niederschlag von Kupferverbindungen der Pentose-Phosphorsäure. Da aber die freigemachte Essigsäure auf den Niederschlag lösend wirkte, wurde die Lösung mit gefällttem Bariumcarbonat versetzt, und die Mischung schnell zum Sieden gebracht und rasch filtriert. Das Filtrat wurde von Spuren Kupfer mittels Schwefelwasserstoff befreit und das Filtrat auf 50 ccm eingedampft. Die Lösung, welche nun ganz frei von Phosphorsäure war, wurde mit 0.4 g Phenylhydrazin in Essigsäure-Lösung versetzt und  $\frac{3}{4}$  Stunden lang auf dem Wasserbade erhitzt. Die Lösung wurde dann von etwaigem Öle abfiltriert und im Eisschrank stehen gelassen. Nach dem Abkühlen schied sich das Osazon als hellgelbe, gelatinöse Masse aus, die unter dem Mikroskope als sehr feine, federartige Nadeln erschien. Diese wurden abgesaugt und aus möglichst wenig pyridinhaltigem Wasser umkrystallisiert. Das Osazon wurde nun ganz rein erhalten. Im Capillarrohr erhitzt, schmolz es scharf gegen 163—164° (korr.). Die Ausbeute betrug 0.060 g.

0.050 g in 4 ccm Pyridin-Alkohol-Gemisch (3 Alkohol-2 Pyridin) drehte im 0.5-dm-Rohre mittels Natriumlicht 0.29°. Das Drehungsvermögen einer gleichen Menge Xylosazon ist 0.08°.

Ganz ohne Schwierigkeiten läßt sich das Osazon aus dem Inosin gewinnen. Um nun aber die Befunde, die man bei der Analyse des Inosins gewinnt, für die Aufklärung der Konstitution der Inosinsäure gültig zu machen, muß man alle Beweise für die Identität der beiden Inosine beibringen. — In Ergänzung zu den gemeinschaftlichen Eigenschaften, welche in der früheren Mitteilung erwähnt sind, wollen wir hier noch über die optischen Eigenschaften der beiden Substanzen berichten. — Nach Haiser und Wenzel besitzt eine ca. 10-prozentige Lösung seines Inosins das Drehungsvermögen  $[\alpha]_D = -49.2^\circ$ . In unsern Versuchen waren wir gezwungen, mit viel verdünnteren

Lösungen zu arbeiten. — 0.0694 g Inosin aus Carnin wurden in 4 ccm Wasser gelöst; Gesamtgewicht der Lösung 4.6020 g; drehte im 0.5-dm-Rohr mit Natriumlicht 0.36 nach links, mithin  $[\alpha]_D = -47.75^\circ$ . 0.0709 g Inosin aus Inosinsäure wurden in 4 ccm Wasser gelöst; Gesamtgewicht der Lösung 4.2145 g; drehte im 0.5-dm-Rohr 0.40° nach links, mithin  $[\alpha]_D = -47.56^\circ$ .

Es liegt nun kein Zweifel mehr an der Identität der beiden Substanzen vor; darum beschlossen wir, zum weiteren Studium der Natur des Zuckerrestes der Inosinsäure als Ausgangsmaterial das Karnin zu verwenden.

#### Die Pentose aus Carnin.

10.0 g Carnin, nach der Methode von Haiser und Wenzel hergestellt, wurden in einem Liter  $1/10$ -n. Schwefelsäure genau eine Stunde lang gekocht. — Länger darf es nicht sein, da schon dann etwas Zersetzung anfängt, und das Krystallisieren des freien Zuckers erschwert wird. Nach dem Abkühlen wurde das Hypoxanthin mit einer heiß gesättigten Silbersulfatlösung vollständig gefällt. — Nach dem Abkühlen wurde abfiltriert und das Silber im Filtrat mittels Schwefelwasserstoff beseitigt. — Die Schwefelsäure wurde dann genau mittels Baryt entfernt und die Lösung, welche nur freie Pentose enthalten sollte, wurde unter vermindertem Druck zu einem farblosen Sirup eingengt. — Der Sirup wurde dann in heißem, absolutem Alkohol aufgenommen und in einer Schale im Vakuumexsiccator über Schwefelsäure zu einem dicken Sirup eingedampft. — Nach fleißigem Reiben mit einem Glasstabe fängt die Krystallisation an, und nach einiger Zeit erstarrt die Masse ganz.

Die Masse wurde dann mit wenig Alkohol angerührt, abgesaugt und mit wenig Alkohol und Äther gewaschen. Die Mutterlauge erstarrte nach dem Eindampfen auch. Die Ausbeute am rohen Produkt ist, wenn das Carnin ein Molekulargemisch von Inosin und Hypoxanthin ist, beinahe quantitativ, also 3 g. — Der Zucker wurde aus heißem absoluten Alkohol umkrystallisiert. Aus dieser Lösung scheidet er sich langsam im Eisschranke in mikroskopischen Platten aus. Im Capillarrohre erhitzt, schmilzt er gegen  $86-87^\circ$  (korr.), was viel zu niedrig für eine Xylose oder Arabinose ist. Durch wiederholtes Umkrystallisieren veränderte sich der Schmelzpunkt nicht. Zur Analyse wurde im Vakuum über Phosphorsäureanhydrid bei  $80^\circ$  erhitzt. Das Gewicht blieb konstant.

0.1287 g Sbst.: 0.1876 g  $\text{CO}_2$ , 0.0762 g  $\text{H}_2\text{O}$ .

$\text{C}_5\text{H}_{10}\text{O}_5$ . Ber. C 40.00, H 6.66.

Gef. » 39.75, » 6.58.

Für die optische Bestimmung wurde in Wasser gelöst.

0.1321 g Sbst. wurden in 3 ccm Wasser gelöst. Gesamtgewicht der Lösung 3.3872 g.

Drehte im 1-dm-Rohr mit Natriumlicht  $0.76^\circ$  nach links. Ohne Berücksichtigung des spezifischen Gewichts:  $[\alpha]_D = -19.5^\circ$ .

Dieser Wert ist ganz anders, als man von *l*-Xylose oder *d*-Arabinose erwarten sollte.

### Darstellung des Phenylsazons.

1 g Zucker, in 50 ccm Wasser gelöst, wurden nach der üblichen Methode mit 2 g Phenylhydrazin, in Essigsäure gelöst, behandelt. Nach dem Erkalten schied sich das Osazon genau wie bei der Inosinsäure als gelatinöse Masse<sup>1)</sup> ab, die unter dem Mikroskop als aus sehr dünnen, verfilzten Nadeln bestehend erschien, welche manchmal zu Rosetten verwachsen waren. Die Ausbeute betrug 1.2 g. — Nach mehrmaligem Umkrystallisieren aus pyridinhaltigem Wasser blieb der Schmelzpunkt bei  $163\text{--}164^\circ$  konstant.

Zur Analyse wurde im Vakuumexsiccator über Schwefelsäure getrocknet.

0.1522 g Sbst.: 0.3480 g  $\text{CO}_2$ , 0.0868 g  $\text{H}_2\text{O}$ .

$\text{C}_5\text{H}_8\text{O}_3(\text{N.NH.C}_6\text{H}_5)_2$ . Ber. C 62.36, H 6.34.

Gef. » 62.01, » 6.38.

Zur Bestimmung der optischen Drehung<sup>1)</sup> wurden 0.0502 g der Substanz in 5 ccm Pyridinalkohol gelöst. Die Drehung war im 0.5-dm-Rohr mit Natriumlicht  $-0.23^\circ$ . Also ist, wenn die Drehung auf 0.2 g Substanz in 10 ccm gelöst berechnet wird,  $\alpha = -0.92^\circ$ . Der aus dem Inosinsäurezucker erhaltene Wert, auch auf dieselbe Konzentration berechnet, gibt ebenfalls  $\alpha = -0.92^\circ$ .

### Das Benzylphenylhydrazon.

Zwei Versuche wurden angestellt, einer mit 0.5 g der Substanz und der zweite mit 1.0 g. Zu diesem Zweck wurde die Pentose in heißem Alkohol gelöst und mit einer alkoholischen Lösung der äquimolekularen Menge von Benzylphenylhydrazin am Rückflußkühler im

<sup>1)</sup> Nachtrag bei der Korrektur. Für die optische Untersuchung des Phenylsazons hatten wir der starken Färbung der Lösung bisher nur 0.05 g in 5 ccm Pyridin Alkohol benutzen können. Seit Einsendung dieser Mitteilung ist es uns aber gelungen, das Osazon so weit zu reinigen, daß die Lösung hell genug war, um in der üblichen Konzentration verwendet zu werden. 0.1006 g in 5 ccm gelöst zeigten im 1-dm-Rohr eine Drehung von  $1.16^\circ$ , was genau mit der für *d*-Arabinose berechneten Drehung übereinstimmt. Da die sonstigen Eigenschaften des Zuckers und seiner Derivate von denen der *d*-Arabinose stark abweichen, so bleiben für unsere Zucker nur zwei Möglichkeiten, nämlich daß wir es mit *d*-Ribose oder mit *d*-Arabinoketose zu tun haben. Für die Entscheidung dieser Frage möchten wir auf den demnächst erscheinenden Artikel verweisen.

Wasserbad erhitzt. — Die Benzylphenylhydrazinlösung wurde frisch bereitet.

Nach dem Erhitzen wurde die klare Lösung mit kaltem Wasser bis zur Entstehung einer Opalescenz versetzt und bei 0° stehen gelassen. Es bildete sich eine halb krystallinische Masse. Die Mutterlauge wurde dekantiert, der Niederschlag in Äther gelöst, und mit Petroläther gefällt. Die Operation wurde nochmals wiederholt. Diese Substanz hatte den Schmp. 116°. Zur weiteren Reinigung wurde die Substanz 2 mal aus Essigäther umkrystallisiert, worauf der Schmelzpunkt konstant blieb. Im Capillarrohr erhitzt schmolz sie scharf gegen 127—128° (korr.).

0.1202 g Sbst. wurden in 3 ccm absolutem Alkohol gelöst. Gesamtgewicht der Lösung 2.7888 g drehte im 0.5-dm-Rohr mit Natriumlicht 0.57° nach links.

Mithin  $[\alpha]_D = -26.46^\circ$ .

Zur Analyse wurde die Substanz über Phosphorsäureanhydrid bei 80° getrocknet.

0.1080 g Sbst.: 0.2610 g CO<sub>2</sub>, 0.0654 g H<sub>2</sub>O.

C<sub>18</sub>H<sub>22</sub>N<sub>2</sub>O<sub>4</sub>. Ber. C 65.45, H 6.65.

Gef. » 65.01, » 6.77.

### Darstellung des Furfurol-Phloroglucides.

In unserer ersten Mitteilung haben wir das Resultat einer Furfurolbestimmung berichtet. Das Experiment wurde genau nach den Angaben von Grund an ausgeführt, und wir hatten erwähnt, daß die Destillation solange fortgesetzt wurde, bis in dem weiteren Destillat kein Furfurol mehr nachweisbar war. Dieser Befund war von Neuberg und Brahn<sup>1)</sup> in Zweifel gestellt. Wir haben nun das Experiment unter Verfolgung aller Maßregeln wiederholt. Die Resultate waren folgende: Aus 0.5010 g Barium-Inosinat haben wir nur 0.0200 g Phloroglucid erhalten. Hr. Dr. Heimrod hatte die Güte, noch eine andere Bestimmung zu machen. Aus 0.6426 g Barium-Inosinat hat er nur 0.0277 g Phloroglucid erhalten. So ist aus dem Barium-Inosinat nur ein Viertel der Menge von Phloroglucid zu erhalten, die man von einer Aldose erwarten sollte.

Bei dem Inosin, das sehr leicht durch Säuren gespalten wird, war das Resultat ganz anders. Aus 0.3089 g Inosin haben wir 0.1373 g Phloroglucid erhalten, was ungefähr einer Aldopentose entspricht.

<sup>1)</sup> Diese Berichte 41, 3376 [1908].





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## Bemerkungen zur Wirkung von Adrenin<sup>1)</sup> auf die Froschpupille.

Von S. J. Meltzer.

Die Benutzung der Froschpupille als Reagens für Adrenalin wird von den Autoren als „Ehrmannsche Methode“ bezeichnet. Es sei mir gestattet, hier den historischen Sachverhalt darzulegen.

In Nummer 11 des XVIII. Bandes des Zentralblattes für Physiologie (27. August 1904) haben Clara M. Auer und ich die Mitteilung gemacht, dass bei Fröschen Einspritzung in den Lymphsack oder Instillation von Adrenalin eine charakteristische Erweiterung der Pupille verursacht. Wir haben daran die Bemerkung angeschlossen, dass die Pupille des Frosches sich als ein geeignetes Reagens für Nebennierenextrakt erweisen dürfte, und zwar haben wir diese Bemerkung gesperrt drucken lassen. Bald darauf erschien unser ausführlicher Artikel im American Journal of Physiology (Vol. XI, 1904, p. 449), auf den wir be-

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<sup>1)</sup> Prof. E. A. Schäfer aus Edinburg hat in den kürzlich gehaltenen Oliver-Sharpey Vorträgen (British Medical Journal 1908, I, p. 1279) den wohlbegründeten Vorschlag gemacht, für das Extrakt der Nebenniere den kurzen, bezeichnenden und unverfänglichen Namen „Adrenin“ zu gebrauchen. Die Bezeichnungen „Adrenalin“, „Suprarenin“, „Hemisin“ usw. bedeuten nur das Extrakt in gewisser Darstellungsweise und haben noch ausserdem einen kommerziellen Beigeschmack.

reits im Zentralblatt verwiesen haben. In diesem Artikel sagen wir in bezug auf Instillation unter anderem: „Finally, in carefully excised eyes, dropping of adrenalin upon the corneal surface brought on promptly a dilatation of the pupil which lasted for many hours“. Am Schluss der Arbeit sagen wir: „We wish to call attention to the fact that the frogs eye, excised or in situ, might prove to be a better reagent than the blood-pressure...“.

Wir haben somit nicht nur die Hauptsache entdeckt, dass nämlich die Froschpupille durch Adrenin erweitert wird, sondern haben auch deutlich genug angegeben, dass dies auch an der Pupille des enukleierten Bulbus geschieht und dass dieses Verhalten der Froschpupille als Reagens für Adrenin verwendbar ist.

Ungefähr ein Jahr später erschien die Arbeit von E h r m a n n<sup>1)</sup>), worauf sein Anspruch als der Urheber der Froschpupillennmethode sich gründet. In einer Anmerkung zu Beginn derselben sagt der Autor: „Auf der Suche nach einem feinen physiologischen Reagens für das Adrenalin wurde ich von Herrn Dr. Oswald Loeb auf die Möglichkeit der Verwertung der damals gerade erschienenen Melzerschen Mitteilung freundlichst hingewiesen.“ E h r m a n n beschreibt dann die Applikation des Adrenalin am enukleierten Bulbus als eine von ihm beobachtete neue Tatsache. In seinem in dieser Wochenschrift (1908, S. 783) abgedruckten Vortrage, worin E h r m a n n von „seiner biologischen Methode“ spricht, kommt der Name Meltzer nicht mehr vor, obschon er darin noch manches andere vorbringt, was von uns her stammt, wie z. B. die Bedeutung des oberen Halsganglions bei den Säugetieren für die Pupillenerweiterung oder die Verlängerung der Kontraktionsdauer der Ohrgefäße nach blosser Durchschneidung des Halssympathicus. Es waren eben diese Studien, welche uns zur Auffindung der Wirkung des Adrenins auf die Froschpupille geführt haben.

Zur Rechtfertigung E h r m a n n s nehme ich an, dass er keine unserer Originalarbeiten gelesen hat. Wie dem auch sei, das steht doch fest, dass die Methode des Nachweises von Adrenalin mittels der Froschpupille auch des enukleierten Bulbus von uns und nicht von E h r m a n n her stammt.

Methode. In unserer Arbeit im American Journal of Physiology haben wir angegeben, dass wir beim normalen lebenden Frosche mittels einer feinen Pipette zwei oder drei Tropfen

<sup>1)</sup> R. E h r m a n n, Archiv für experimentelle Pathologie und Therapie 1905, Bd. 53, Seite 97.

Adrenalin zwischen Lid und Auge eingeträufelt haben, wovon natürlich nur ein kleiner Bruchteil darin verblieb. Bei dieser Methode wird die Pupille oft schon nach drei Minuten charakteristisch erweitert. Die Erweiterung hält oft 24 Stunden an, kehrt aber schliesslich zur normalen Weite zurück. Diese Methode bietet den Vorteil, dass man an demselben Tiere und an demselben Auge den Versuch mehrfach ausführen und demnach auch Vergleiche anstellen kann. In dieser Weise habe ich z. B. das normale Adrenin (Adrenalin) mit dem von Stolz künstlich hergestellten (Aethylaminoketon) an demselben Tiere mehrfach miteinander verglichen und gefunden, dass das künstliche Produkt viel weniger wirksam ist. Wir haben ferner Instillationen auch bei Tieren ausgeführt, deren Halsmark durchtrennt, oder deren Gehirn, oder auch deren ganzes Zentralnervensystem zerstört war. Endlich haben wir die Instillation an abgeschnittenen Köpfen und an enukleierten Bulbi studiert. Im letzteren Falle wurde das Adrenalin einfach auf die Cornea geträufelt.

Seitdem habe ich verschiedene Methoden ausprobiert, welche die Ausführung der Reaktion einfach und ökonomisch ermöglichen sollen. Ich werde hier nur eine Methode erwähnen, welche in mehrfacher Hinsicht praktisch zu werden verspricht.

Man schneidet mit einer Schere rasch den Kopf ab, etwa am vorderen Rande der Gehörmembranen (zerstört natürlich aus humanitären Gründen Gehirn und Rückenmark), trägt die unteren Lider ab und spaltet dann den Kopf der Länge nach. Dann befestigt man jeden Teil an einem Korkstückchen mittels einer Stecknadel in solcher Weise, dass die Cornea gerade nach oben gerichtet ist. Vorher schneidet man sich einige Ringe von etwa 3 mm Höhe von einem Gummischlauche zurecht, dessen Lumen etwa der Grösse der vorderen Augenfläche entspricht. Man setzt nun mittels einer Pinzette einen solchen Ring<sup>1)</sup> auf das Auge in einer solchen Weise, dass die Pupille etwa in der Mitte des Lumens zu liegen kommt, und träufelt in die Mitte des Lumens einen Tropfen ein. In der Regel läuft nichts ab; aber wenn auch etwas davon entkommt, so wird doch das meiste davon durch Kapillarität zurückgehalten.

Ich habe mit dieser Methode, bei der doch so wenig Flüssigkeit zur Verwendung kommt, oft einen rascheren Erfolg erzielt, als wenn der ganze Bulbus in viel Flüssigkeit gebadet wurde. (Der Druck, welcher durch die Oberflächenspannung der durch die Kapillarität sich bildenden Membranen ausgeübt wird, be-

<sup>1)</sup> Der untere Rand des Ringes kann ein wenig mit Oel (oder Vaseline) bestrichen werden.

schleunigt wahrscheinlich den osmotischen Vorgang.). Man kann den Ring mehrfach abheben und wieder aufsetzen, ohne dass die Flüssigkeit entweicht. Uebrigens kann man auch den Ring in die Flüssigkeit eintauchen und in dieser Weise den Tropfen aufnehmen, was für einen hervorquellenden Blutstropfen namentlich vorzuziehen wäre.

**Charakteristische Merkmale.** Die Adreninreaktion besteht nicht bloss in einer Erweiterung der Pupille, sondern auch in der Starre, in der Reaktionslosigkeit auf Licht und in der Abrundung der Pupillaröffnung. Wenn die Reaktion am lebenden Frosche vorgenommen wird, fällt die Starre der mit Adrenin behandelten Pupille auf. Während die normale Pupille mit Aufregungen und Bewegungen, namently Befreiungsanstrengung auch bei gleichbleibender Belichtung, gewisse Veränderlichkeiten zeigt, bleibt die adrenisierte Pupille unveränderlich starr. — Noch auffälliger ist die Reaktionslosigkeit auf Licht, was auch an der Pupille des exzidierten Bulbus beobachtet werden kann.

Die durch Adrenalin bewirkte Erweiterung der Pupille kennzeichnet sich auch durch eine Abrundung. In der normalen Pupille ist die Längsachse stets wesentlich grösser als die Höhenachse. Die erste Wirkung des Adrenalins besteht zunächst wesentlich in einer Vergrösserung des Höhendurchmessers, sodass sich die Pupille mehr und mehr abrundet. Die endliche Vergrösserung betrifft beide Durchmesser. Eine Erweiterung der Pupille, wobei beide Durchmesser ihre ursprünglichen Proportionen behalten, stammt wohl nicht vom Adrenalin her.

Die Pupillenerweiterung, welche durch Adrenalin bewirkt wird, ist keine komplette, d. h. es bleibt von der Iris immer ein beträchtlicher Saum zurück, der durch keine weitere Instillation oder Injektion vermindert werden kann. Die Erweiterung hat demnach eine obere Grenze, welche jedoch individuell schwankt. Unterhalb dieser Grenze ist die Erweiterung im allgemeinen proportional der Grösse der Dose oder der Konzentration der benutzten Lösung. Das Intervall zwischen der Instillation (oder Injektion) und dem Eintritt der Reaktion ist umgekehrt proportional der Dose oder Konzentration. Mit anderen Worten: je kleiner die Dose oder je grösser die Verdünnung, um so kleiner ist die Erweiterung, und um so später tritt sie ein.

Mit der zunehmenden Verdünnung nimmt die Reaktion allmählich an Schärfe ab. Ehrmann gibt an, dass er bei Konzentrationen, die noch dünner waren als 1:10 000 000, eine

Erweiterung der Pupille feststellen konnte. Er versucht sogar, aus der Feststellung der unteren Grenze ein Mittel zu schaffen, das uns ermöglichen soll, mit Hilfe der Froschpupille ganz geringe Mengen von Adrenin quantitativ zu bestimmen. Ich kann ihm da nicht folgen und gebe demgegenüber folgendes zu bedenken. Zwischen einer Konzentration, die eine sichere Reaktion gibt, und einer solchen, bei der man sicher ist, dass sie keine Reaktion gibt, ist der Uebergang kein scharfer, abrupter. Im Gegenteil, man kann oft schon bei einer Verdünnung von nur 1:150 000 ernstlich im Zweifel sein, ob eine Veränderung an der Pupille da sei. Andererseits kann man manchmal noch bei einer Verdünnung von 1:10 000 000 wirklich den Eindruck haben, dass eine gewisse Erweiterung eingetreten ist. Kontrollen können uns da garnicht helfen. Die untere Grenze der Reagierbarkeit schwankt von Frosch zu Frosch, und oft ist sie auch verschieden an den beiden Augen desselben Frosches. Dann sind oft die Pupillen beider enukleierten Bulbi desselben Frosches auch normalerweise verschieden weit. Ferner habe ich beobachtet, dass auch eine Ringersche Lösung gelegentlich eine geringe Erweiterung verursacht. (Die Kontrollen werden von den Untersuchern nur mit „physiologischer Kochsalzlösung“ gemacht, was unrichtig ist; und dazu ist noch diese Kochsalzlösung gewöhnlich diejenige, welche jetzt in biologischen Laboratorien üblich ist, nämlich 0,85% oder 0,92%, was für Frösche hypertönische Lösungen darstellt.)

Auf Grund meiner Erfahrungen bin ich der Ansicht, dass die untere Grenze der Wirksamkeit des Adrenins auf die Froschpupille viel zu schwankend, zu unzuverlässig ist, um darauf eine Messungsmethode konstruieren zu können. Glücklicherweise sind die interessanten Funde von Wiesel und Schur, von Eichler, von Reicher u. a. nur auf Grund von qualitativen, nicht von quantitativen Vergleichen gemacht worden. Normales Serum verursacht gar keine Erweiterung der Pupille, während die von den genannten Autoren untersuchten pathologischen Sera eine unzweifelhafte Erweiterung bewirkt haben.

Im übrigen möchte ich anraten, sich nicht auf die Erweiterung allein zu verlassen, sondern auch die Abrundung der Pupille und deren Reaktionslosigkeit zum Lichte als charakteristisches Merkmal in Betracht zu ziehen. Ferner möchte ich dringend anraten, namentlich wenn es sich um Feststellung von neuen, wichtigen Tatsachen handelt, dass das am enukleierten Bulbus gewonnene Resultat, wo es geht, noch am

ganzen Frosche durch Einspritzung der untersuchten Flüssigkeit in den Lymphsack zu kontrollieren. Der Grund dafür wird im nächsten Absatze ersichtlich werden. Zu diesem Zwecke soll man das Gehirn des Frosches zerstören, wodurch beide Pupillen sehr klein werden, und der eine Bulbus soll dann exzidiert und zur Kontrolle aufbewahrt werden. Nach meinen Erfahrungen genügt dafür 1 ccm Adrenalin in einer Verdünnung von 1:100 000 (= 0,01 ccm des unverdünnten Adrenalins!).

**Brenzkatechinwirkung.** Waterman und Boddaert haben in dieser Wochenschrift (1908, S. 1102) kürzlich angegeben, dass die Pupille des Frosches auch durch Brenzkatechin und deren Derivate erweitert wird, und bezweifelten darum, dass die von den Autoren angegebene mydriatische Wirkung gewisser Sera wirklich von im Blute anwesendem Adrenin herstamme. Ich habe die Wirkungen von Brenzkatechin, Resorcin und Salizylsäure geprüft. In meinen Erfahrungen sind die Erweiterungen der Pupille, welche durch Resorcin und Salizylsäure entstehen, so gering, dass sie mit der Wirkung von Adrenalin durchaus nicht verglichen und nicht verwechselt werden können. Brenzkatechin hatte in der Verdünnung, wie sie Waterman und Boddaert angeben (1:500), in meinen Versuchen gar keine wahrnehmbare Wirkung ausgeübt. Dagegen war bei Benutzung einer starken Lösung (5%) in der Tat eine beträchtliche Erweiterung der Pupille eingetreten. Die Wirkung unterschied sich jedoch in zwei Punkten: in allen beobachteten Fällen war die Pupille nie rund, sondern blieb oval, und dann war das vordere Kammerwasser stets stark getrübt, was bei Adrenalisierung nie vorkommt. Ein schlagender Unterschied zwischen den Wirkungen beider Substanzen kam jedoch zum Vorschein, als die Einspritzung zur Kontrolle benutzt wurde. Eine Einspritzung von Brenzkatechin in den Lymphsack des Frosches verursacht (ausser den bekannten fibrillären Zuckungen und Konvulsionen) nicht eine Erweiterung, sondern eine starke Verengerung der Pupillen, so stark, wie man sie nur nach Zerstörung des Nervensystems sieht.

**Wirkung des Hypophysensaftes.** Bei der Deutung der mydriatischen Wirkung gewisser Sera als Adreninwirkung müsste jedoch die von W. Cramer<sup>1)</sup> neuerdings mitgeteilte Beobachtung ernstlich in Erwägung gezogen werden, dass nämlich das Extrakt der Hypophyse eine

<sup>1)</sup> W. Cramer, Quarterly Journal of Experimental Physiology, 1908, Vol. 1, S. 189.

Erweiterung der Froschpupille bewirkt. Die Zunahme des Höhendurchmessers scheint da beträchtlich zu sein. Nach Cramer tritt die Wirkung des Hypophysensaftes etwas später ein als die des Adrenalins, wirkt aber nachhaltiger. Sehr verdünnte Lösungen können noch nach 16 Stunden wirksam werden. Bedeutsam mag ferner die Angabe sein, dass nur der Saft des hinteren Teiles erweiternd wirkt, also des Teiles, der nach Schäfer und Herring auch das diuretisch wirksame Element enthält.<sup>1)</sup> Ueber Einspritzungen mit diesem Saft bei Fröschen und anderen Tieren liegen noch keine Versuche vor.

<sup>1)</sup> Schäfer und Herring, Philos. Transact., Series B, 1906, Vol. 77, S. 571.



## Über die Hefenucleinsäure.

Von

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Über die Zusammensetzung der Hefenucleinsäure liegen ältere Arbeiten von Kossel<sup>1)</sup> und die in neuerer Zeit in Schmiedeberts Laboratorium ausgeführten Untersuchungen von Herlant<sup>2)</sup> und Boos<sup>3)</sup> vor.

Nach diesen Untersuchungen unterscheidet sich die Hefenucleinsäure von allen anderen Nucleinsäuren tierischen und pflanzlichen Ursprungs. — Die empirische Formel weicht von der anderer Nucleinsäuren bedeutend ab, wie das aus der folgenden Zusammenstellung ersichtlich ist:

Spermanucleinsäure	C	H	N	O	P
(nach Schmiedeberg <sup>4)</sup> )	40	56	14	26	4
Thymusnucleinsäure					
(nach Steudel <sup>5)</sup> )	40	56	15	26	4
Milznucleinsäure					
(nach Levene und Mandel <sup>6)</sup> )	54 <sup>7)</sup>	71	20	37	5

<sup>1)</sup> A. Kossel, Arch. f. Anat. u. Physiol. 1891, 181; 1893, 159; 1894, 199.

<sup>2)</sup> Herlant, Arch. f. experim. Pathol. u. Pharmakol. 44, 159, 1900.

<sup>3)</sup> Boos, Arch. f. experim. Pathol. u. Pharmakol. 55, 16—20, 1906.

<sup>4)</sup> Arch. f. experim. Pathol. u. Pharmakol. 57, 309, 1907.

<sup>5)</sup> Zeitschr. f. physiol. Chem. 46, 332, 1905.

<sup>6)</sup> Ber. d. Deutsch. chem. Ges. 41, 1905, 1908.

<sup>7)</sup> Sollte es sich herausstellen, daß die komplizierteren tierischen Nucleinsäuren eine Tetraphosphorsäure enthalten, so würde man nicht C<sub>40</sub>, sondern C<sub>43</sub> annehmen und die Formel in C<sub>43</sub>H<sub>55</sub>N<sub>5</sub>O<sub>31</sub>P<sub>4</sub> abändern müssen.

Weizenembryonucleinsäure	C	H	N	O	P
(nach Osborn u. Harris <sup>1)</sup> )	42	62	16	31	4
Hefenucleinsäure	17	26	6	14	2
(nach Kossel <sup>2)</sup> )	25	36	9	20	3
(nach Boos <sup>3)</sup> )	36	52	14	24	4

Auch in der Natur des Kohlenhydrates soll diese Substanz sich von den anderen unterscheiden, da nach Kossel bei der Hydrolyse der Hefenucleinsäure zwei Osazone von den respektiven Schmelzpunkten 204 bis 205 und 150° C sich darstellen lassen. Im Gegensatz hierzu gelingt es nicht, ein Osazon nach der Spaltung der Thymonucleinsäure zu erhalten, und aus der Triticonucleinsäure von Osborn und Harris gelang es nur, eine Pentose zu gewinnen.

Über das Verhältnis der Basen zueinander und über die Anordnung, in welcher die Komponenten miteinander verknüpft sind, liegen keine Angaben vor.

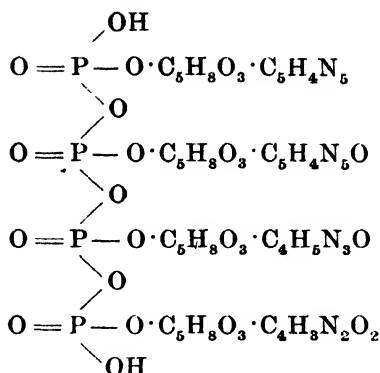
Die vorliegende Untersuchung wurde unternommen, um die Stellung der Hefenucleinsäure zu den anderen Nucleinsäuren aufzuklären. Es stellte sich dabei heraus, daß der Substanz am besten die empirische Formel  $C_{38}H_{50}N_{15}P_4O_{29}$  zukommt, und daß sie in ihrer Zusammensetzung mit der Triticonucleinsäure von Osborn und Harris wahrscheinlich identisch ist. Die vier Basen kommen in äquimolekularem Verhältnis vor. Die Substanz enthält scheinbar nur eine Pentose. Es läßt sich bei der alkalischen Hydrolyse, gerade so wie bei der Inosinsäure,<sup>4)</sup> Phosphorsäure abtrennen, ohne eine reduzierende Substanz zu erhalten, man gelangt also zu Komplexen, die aus Zucker und Basen zusammengesetzt sind, welche die Fehling'sche Lösung nicht reduzieren, wohl aber nach Hydrolyse mit Mineralsäuren. Sie sind ziemlich resistent gegen Alkalien, viel empfindlicher gegen Säuren. Sie besitzen also die Eigenschaften von Glucosiden. Außerdem gelang es, bei der Hydrolyse mittels verdünnter Schwefelsäure eine Substanz zu erhalten, die scheinbar einen Komplex Phosphorsäure-Pentose-Uracil darstellte. Auf Grund dieser Befunde kann man sich die Zusammensetzung etwa nach dem folgenden Schema erklären:

<sup>1)</sup> Zeitschr. f. physiol. Chem. 36, 85, 1902.

<sup>2)</sup> Arch. f. Anat. u. Physiol. 1891, 181.

<sup>3)</sup> Arch. f. experim. Pathol. u. Pharmakol. 55, 16—20, 1906.

<sup>4)</sup> Ber. d. Deutsch. chem. Ges. 41, 2704, 1908.



Dasselbe gilt wahrscheinlich auch für die Triticonucleinsäure von Osborn und Harris.

### Experimenteller Teil.

**Darstellung und Eigenschaften.** Die Substanz wurde aus einem käuflichen Präparate, das noch biurethaltig war, dargestellt. Zu diesem Zwecke wurde die käufliche Säure mittels ganz wenig Ammoniakwasser aufgelöst und mittels Eisessig gefällt. Ein mäßiger Überschuß von Eisessig hält die Nucleinsäure in Lösung, und nur ein ganz großer Überschuß schlägt sie nieder. So z. B. sind 4 kg Eisessig nötig, um die Substanz aus einer konzentrierten Lösung von 50 g Nucleinsäure zu fällen. Der Niederschlag läßt sich sehr gut über Seide mittels einer Saugpumpe filtrieren. Mit Alkohol und Äther gewaschen und getrocknet stellt die Substanz ein schneeweißes Pulver dar. Die Substanz ist auch in Mineralsäuren unlöslich, geht aber bei etwa 24stündigem Stehen mit 2% Schwefelsäure in Lösung, scheinbar ohne Hydrolyse. Beim Neutralisieren mit Alkalien geht die Lösung nicht in eine Gallerte über, wie das bei der Thymonucleinsäure der Fall ist. Die Substanz ist ziemlich resistent gegen Kochen mit verdünnten Alkalien und empfindlicher gegen Kochen mit Säuren. Sie ist optisch aktiv, und das Drehungsvermögen ändert sich mit der Konzentration der Lauge, in welcher die Substanz aufgelöst ist, wie das W. Jones<sup>1)</sup> für Thymonucleinsäure beobachtet hat.

<sup>1)</sup> W. Jones, Journ. of Biolog. Chem. 5, 1, 1908.

1,0 g der Substanz in 25 ccm 10%igen Ammoniakwassers gelöst, hatte das spezifische Gewicht 0,989 und das Drehungsvermögen von  $+1,23^\circ$  bei  $20^\circ$  und  $l = 86,5$  mm,

$$[\alpha]_D^{20} = +35,94^\circ.$$

0,5 g der Substanz in 10 ccm 12,5%igen Ammoniakwassers gelöst, hatte das Gesamtgewicht 9,1276 g, spezifisches Gewicht 0,996 und das Drehungsvermögen von  $+1,06^\circ = 20^\circ \text{ C}$  und  $l = 50,0$  mm.

Mithin  $[\alpha]_D^{20} = +38,84^\circ.$

1,0 g der Substanz, in 20 ccm von  $\frac{n}{1}$ -NaOH gelöst, hatte das Gesamtgewicht 20,662 g, das spezifische Gewicht 1,06 und das Drehungsvermögen von  $+0,20^\circ$  bei  $20^\circ \text{ C}$  und  $l = 50,0$  mm.

Mithin  $[\alpha]_D^{20} = +7,8^\circ.$

Die elementare Zusammensetzung der Substanz war folgende:

0,1546 g der Substanz gaben 0,1980 g  $\text{CO}_2$  und 0,0642 g  $\text{H}_2\text{O}$ , mithin  $\text{C} = 34,93\%$ ,  $\text{H} = 4,30\%$ .

0,1734 g der Substanz gaben 0,2226 g  $\text{CO}_2$  und 0,0702 g  $\text{H}_2\text{O}$ , mithin  $\text{C} = 35,01\%$ ,  $\text{H} = 4,52\%$ .

0,2162 g der Substanz verbrauchten bei einer Stickstoffbestimmung nach Kjeldahl 23,5 ccm  $\frac{n}{10}$ - $\text{H}_2\text{SO}_4$ , mithin  $\text{N} = 15,21\%$ .

0,6450 g der Substanz gaben beim Schmelzen 0,1987 g  $\text{Mg}_2\text{P}_2\text{O}_7$ , mithin  $\text{P} = 8,6\%$ .

Für die angenommene Formel  $\text{C}_{38}\text{H}_{50}\text{N}_{15}\text{P}_4\text{O}_{20} + \text{C}_2\text{H}_4\text{O}_2$ .

	C	H	N	P
Berechnet	35,18%	4,00%	15,29%	9,08%
Gefunden	34,97%	4,41%	15,21%	8,6%

### Purinbasen.

Die Purinbasen waren in mehreren Versuchen dargestellt, obwohl diese nicht alle zum Zwecke der Bestimmung des Verhältnisses dieser Basen unternommen wurden. Man gewinnt aus allen Experimenten den Eindruck, daß die Basen im Moleküle der Nucleinsäure in äquimolekularen Quantitäten vorkommen. Die Ausbeute an Purinbasen betrug in den meisten Experimenten nicht ganz 20% des Gesamtgewichtes vom Aus-

gangsmaterial, während nach der angenommenen Formel man 21% erwarten dürfte. Diese Übereinstimmung ist nicht ganz unbefriedigend, wenn man alle möglichen Fehlerquellen, die bei solchen Experimenten vorkommen, in Betracht zieht. Als ein Beispiel sei folgender Versuch erwähnt:

10,0 g der Substanz wurden mit 200,0 ccm 1% Schwefelsäure 10 Stunden lang im Autoklaven bei 125° C erhitzt. Am Ende der Erhitzung war die Lösung hellbraun und enthielt Spuren von Melanin. Beim Abkühlen schieden sich keine Purinbasen aus, was in den Experimenten der Fall war, in welchen die Verdünnung nicht den Grad dieses Versuches betrug. Zu der kalten Lösung wurde eine Lösung von 15,0 g Silbersulfat in etwa 30 ccm konzentrierter Schwefelsäure zugegeben. Es zeigte sich, daß dann ein Überschuß an Silbersulfat in Lösung war. Die Mischung wurde bei — 1° C 48 Stunden lang stehen gelassen. Der Niederschlag von Silberpurinen wurde dann mittels Salzsäure von Silberpurinen möglichst genau befreit, und aus der Lösung 1,0 g Guanin und 2,0 g Adeninpikrat erhalten.

Zur Identifizierung der Basen wurden sie in größeren Quantitäten dargestellt, so daß mehrmalige Reinigung möglich war. Zu diesem Zwecke wurden 80,0 g der Säure mit einem Liter 2%ige Schwefelsäure am Rückflußkühler im Ölbad bei 125° C 4 Stunden lang erhitzt. Beim Abkühlen schied sich der größere Teil der Basen aus. Mit dem Filtrate wurde wie im vorigen Experimente verfahren. Die Sulfate der zwei Basen wurden durch Auskochen mit heißem Wasser getrennt, wobei man das unlösliche, freie Guanin bekommt, während Adeninsulfat in Lösung bleibt und beim Eindampfen der Lösung wieder auskristallisiert. Nach einigem Wiederholen der Operation bekommt man reines Adeninsulfat und reines Guanin. Die Präparate wurden lufttrocken analysiert.

#### Analyse des Guanins.

0,1752 g der Substanz gaben 0,2544 g CO<sub>2</sub> u. 0,0565 g H<sub>2</sub>O.

	C	H
Für C <sub>5</sub> H <sub>5</sub> N <sub>5</sub> O berechnet	39,73%	3,30%
gefunden	39,60%	3,60%

## Analyse des Adeninsulfats.

0,1856 g der Substanz gaben 0,1986 g  $\text{CO}_2$  und 0,0646 g  $\text{H}_2\text{O}$

	C	H
Für $(\text{C}_6\text{H}_5\text{N}_5)_2\text{H}_2\text{SO}_4 + 2\text{H}_2\text{O}$ berechnet	29,70%	3,96%
gefunden	29,18%	3,89%

## Pyrimidinbasen.

Schon in früheren Arbeiten von Kossel und Steudel<sup>1)</sup> und von mir<sup>2)</sup> wurde festgestellt, daß zwei Pyrimidinbasen, Uracil und Cytosin, im Moleküle der Hefenucleinsäure vorkommen. Es wurde nun festgestellt, daß das Cytosin zu den Purinbasen in äquimolekularem Verhältnisse vorkommt. Das Uracil konnte nicht in derselben Proportion erhalten werden. Dieses rührt aber von der großen Löslichkeit der Substanz her. Es bleibt aber nichts anderes übrig, als dieses Verhältnis anzunehmen, wenn das der anderen drei Basen erwiesen ist. Die beiden Pyrimidinbasen konnten aus der Nucleinsäure auch nach der Entfernung der Purinbasen erhalten werden.

Für die quantitative Bestimmung der Pyrimidinbasen wurden 10,0 g der lufttrockenen Substanz mit 40 cm 25%iger Schwefelsäure 4 Stunden lang im Autoklaven auf 175° C erhitzt. Die resultierende, dunkelbraune Flüssigkeit wurde von Phosphorsäure und Schwefelsäure mittels Barytwasser und von dessen Überschuß mittels Schwefelsäure befreit. Das Filtrat und Waschwasser wurde zu einem kleinen Volumen, etwa auf 200 cm, bei vermindertem Drucke eingedampft, und heiß mit einer wässerigen Lösung von Pikrinsäure versetzt. Es bildete sich ein flockiger amorpher Niederschlag. Dieser wurde abfiltriert, und das klare Filtrat zu einem kleinen Volumen bei vermindertem Druck eingedampft und der Krystallisation überlassen. Man erhielt auf diese Weise 3,0 g von Roh-Cytosinipikrat, welches bei der Analyse einen Gehalt von  $\text{C} = 32,5\%$  und  $\text{H} = 2,46\%$  ergab. Nach zweimaligem Umkrystallisieren erhielt man ein reines Cytosinipikrat.

<sup>1)</sup> Zeitschr. f. physiol. Chem. 38, 51, 1903.

<sup>2)</sup> Zeitschr. f. physiol. Chem. 39, 6, 1903.

0,0980 g der Substanz (bei vermindertem Druck über Phosphorpentoxyd bei 108° getrocknet) gaben 0,1260 g CO<sub>2</sub> und 0,0238 g H<sub>2</sub>O

für C<sub>4</sub>H<sub>5</sub>N<sub>3</sub>O · C<sub>6</sub>H<sub>2</sub>(NO<sub>2</sub>)<sub>3</sub>OH

	C	H
Berechnet	35,29%	2,35%
gefunden	35,06%	2,58%

Die Theorie verlangte aus 10,0 g Nucleinsäure eine Ausbeute von etwa 0,8 g Cytosin. Der Befund von 3 g des rohen Cytosin-pikrates stimmt mit der verlangten Zahl gut überein.

Die Mutterlauge des Cytosin-pikrates wurde von Pikrinsäure befreit und dann mit einer Silbernitratlösung und Barytwasser behandelt. Es bildete sich ein Niederschlag; dieser wurde in Wasser suspendiert, mit Schwefelwasserstoff zersetzt und das Filtrat von Silbersulfid bei vermindertem Druck eingedampft. Beim Stehen bildete sich ein Niederschlag, der mikroskopisch aus Globuliten bestand; er konnte wegen der mangelhaften Ausbeute nicht weiter gereinigt werden.

Die Substanz wurde aber in einem anderen Experimente erhalten und identifiziert. Es wurden nämlich etwa 80,0 g von der käuflichen Nucleinsäure in 1 l 20%iger Schwefelsäure am Rückflußkühler 4 Stunden lang bei 125° C erhitzt. Aus der Lösung wurden die Purine mittels Silbersulfat entfernt. Ein Teil des Filtrates wurde von Silber und Schwefelsäure befreit, zu einem kleinen Volumen eingedampft, Schwefelsäure bis zu einem Gehalt von 25% zugesetzt und weiter wie im vorigen Experimente verfahren. Man erhielt dabei reines Uracil.

0,2009 g der Substanz gaben 0,3138 g CO<sub>2</sub> und 0,0656 g H<sub>2</sub>O

	C	H
Für C <sub>4</sub> H <sub>4</sub> N <sub>2</sub> O <sub>2</sub> berechnet	42,82%	3,59%
gefunden	42,61%	3,65%

Es folgt also, daß auch in dieser Nucleinsäure, wie in der von Osborn und Harris, das Uracil ein primäres Spaltungsprodukt darstellt.

### Kohlenhydratgruppe.

Im Gegensatz zu den Nucleinsäuren der tierischen Gewebe läßt sich aus der Hefenucleinsäure leicht ein Kohlenhydrat

abspalten. Schon nach kurzem Erhitzen mittels verdünnten Mineralsäuren bekommt man eine Lösung, welche mit Fehling'scher Lösung einen Niederschlag nicht nur von Purinkupferoxydul, sondern auch von freiem Kupferoxydul bildet. Die Nucleinsäure gibt eine starke, positive Orcinprobe auf Pentose, aber, wie schon Mandel und Neuberg<sup>1)</sup> gefunden haben, eine negative mit Naphthoresorcin. Der leicht abspaltbare Zucker erwies sich als eine Pentose; aber über die genaue Natur des Zuckers kann man sich mit Sicherheit nicht aussprechen. Die Pentose war als Phenyl-osazon identifiziert. Diese wurde auf folgende Weise erhalten:

80,0 g der käuflichen Nucleinsäure wurden mit 1 l 2%iger Schwefelsäure am Rückflußkühler im Ölbade 4 Stunden lang auf 125° C erhitzt. Die Purinbasen wurden mittels Silbersulfat entfernt. Aus dem Filtrat hiervon wurden die Zwischenprodukte der Hydrolyse mittels Silber und Barytlösung entfernt und das Filtrat dieses Niederschlages von Silber und Barium befreit. Die so erhaltene Lösung wurde auf 220 ccm eingedampft. 1,5 ccm reduzierten 0,08742 Kupfer, enthielten also etwa 0,052 Zucker; und die totale Ausbeute an Zucker betrug etwa 7,5 g. 80 ccm der ursprünglichen Lösung wurden mit einer Lösung von 10 g Phenylhydrazin in Eisessig auf dem Wasserbade erhitzt. Das Osazon wurde aus wenig Wasser, welches pyridinhaltig war, umkrystallisiert und zur Analyse gebraucht. Es wurde zuerst im Vakuumexsiccator über Schwefelsäure und dann bei 108° C unter vermindertem Druck über Phosphorpentoxyd getrocknet. Es hatte einen Schmelzpunkt von 162° C.

0,0688 g der Substanz gaben 0,1578 g CO<sub>2</sub> und 0,0398 g H<sub>2</sub>O

für C<sub>17</sub>H<sub>20</sub>N<sub>4</sub>O<sub>3</sub>

	C	H
Berechnet	62,20%	6,09%
Gefunden	62,55%	6,47%

0,2000 g der Substanz wurden in einem Gemisch von 6 ccm Alkohol und 4 g Pyridin gelöst. Im 50,0 mm langen Rohre drehte die Lösung — 0,42°, Xylosazon sollte — 0,12° drehen, Arabinosazon + 0,55°. Um den Apparat und die Lösungsmittel zu kontrollieren, wurden reines Xylosazon und Arabinosazon auf ihr Drehungsvermögen untersucht, und man erhielt dabei

<sup>1)</sup> Diese Zeitschr. 13, 151, 1908.

die theoretischen, von Neuberg<sup>1)</sup> angegebenen Zahlen. Es ist nun nicht klar, ob die Abweichung im Drehungsvermögen des vorliegenden Osazones von denen der l-Xylose oder l-Arabinose auf Verunreinigungen beruht. Da nach allen Angaben über die Pentosen der Nucleinsäuren man hier eine l-Xylose erwarten sollte, wurde es versucht, die Xylonsäure zu erhalten. Dieses allein darf aber nicht der Annahme einer Pentose widersprechen, da nach vorliegenden Erfahrungen (es nicht gelingt, das Cadmiumsalz bei der Anwesenheit von Spaltungsprodukten der Eiweißkörper und von Nucleinbasen zu erhalten<sup>2)</sup>). Auch aus dem Drehungsvermögen der zuckerhaltigen Lösung selbst konnte man nicht mit absoluter Sicherheit auf die Natur der Pentose schließen. So drehte die Lösung, welche nach dem Reduktionsvermögen 1% Zucker enthielt, im 50 mm-Rohr bei 20° C  $-0,07^\circ$ . Eine l-Xyloselösung von entsprechender Konzentration sollte  $-0,09^\circ$  drehen. Die Lösung enthielt aber noch Spuren von phosphorhaltigen Substanzen, welche einen gewissen Einfluß auf die Drehung des Zuckers ausüben konnten.

Im Gegensatz zu den Angaben von Kossel läßt sich außer der Pentose kein anderer Zucker nachweisen. Bei der Hydrolyse mittels starker Schwefelsäure läßt sich zwar mit Äther eine Substanz ausziehen, die ein Silbersalz bildet, doch war der Silbergehalt des Salzes für Lävulinsäure zu hoch. Die Lävulinsäure nämlich verlangte 48,43% Ag, während das gefundene Salz 53,40% Ag enthielt. Dabei war die Ausbeute aus der Substanz sehr gering.

Zur quantitativen Bestimmung der Pentose wurde eine Furfuroldestillation vorgenommen. Das Destillat wurde in zwei Fraktionen aufbewahrt. Die ersten 400 ccm des Destillates stellte die erste Fraktion vor, und die folgenden 200 ccm die zweite. Aus der zweiten Fraktion ließ sich kein Furfurolphloroglucid darstellen. Die Ausbeute an Phloroglucid aus 0,6159 g der Substanz betrug 0,1580 g, was 25% Xylose entspricht. Berechnet für die angenommene Zusammensetzung der Hefenucleinsäure müßte man etwa 44% erwarten. Es scheint aber mit sehr großen Schwierigkeiten verbunden zu sein, bei der Destillation mit Mineralsäuren die berechnete Menge Furfurol

<sup>1)</sup> Ber. d. Deutsch. chem. Ges. 32, 3384, 1899.

<sup>2)</sup> Neuberg, *ibid.* 35, 1473, 1902.

aus mit Phosphorsäure gepaarten Pentosen zu erhalten. Auch hinsichtlich der Ausbeute an Furfurol ähnelt die Hefenucleinsäure der aus den Weizenembryonen erhaltenen Substanz von Osborn und Harris.

### Alkalische Hydrolyse.

Es sind von Schmiedeberg und Alsberg<sup>1)</sup> bei der Spaltung der Nucleinsäuren mittels Alkalien Komplexe, die scheinbar aus Kohlenhydraten und Basen bestanden, erhalten worden. Neulich ist es Jacobs<sup>2)</sup> und mir gelungen, bei der Spaltung der Inosinsäure das Glucosid Inosin zu erhalten. Da diese Substanzen für die Aufklärung der Nucleinsäuren von großer Wichtigkeit sind, so wurde es versucht, sie aus der Hefenucleinsäure darzustellen. 4,0 g der Substanz waren in wenig Wasser aufgenommen, durch Zusatz von normaler Natronlauge diese Substanz in Lösung gebracht und die Zugabe von Natronlauge fortgesetzt, bis die Lösung gegen Phenolphthalein genau alkalisch geworden war. Die Lösung, welche 22 ccm betrug, wurde in einem Einschmelzrohre 8 Stunden lang auf 125 bis 140° C erhitzt. Es resultierte eine hellbraune Lösung, welche keine freien Purinbasen enthielt, Fehlingsche Lösung nicht reduzierte, aber freie Phosphorsäure enthielt. Das optische Drehungsvermögen war nach dem Erhitzen in diesem Falle stark herabgesetzt. 6 ccm dieser Flüssigkeit wurden mit 20 ccm kaltem, ammoniakhaltigem Wasser in Kältemischung gut abgekühlt, mit Salpetersäure angesäuert, mit Molybdänsäure versetzt und über Nacht bei 1° C stehen gelassen. Der Niederschlag wurde abfiltriert, unter starkem Abkühlen in Ammoniakwasser gelöst und mit Salpetersäure gefällt. Dieser Niederschlag auf übliche Weise weiter behandelt, betrug 0,0352  $\text{Mg}_2\text{P}_2\text{O}_7$ . Mithin wurden aus den 4,0 g Nucleinsäure 0,0323 g, welche etwa 10% der Gesamt-Phosphorsäure entsprechen, abgespalten, ohne die Purin-glucoside zu hydrolysieren.

Die Bemühungen, diese Glucoside von den phosphorhaltigen Substanzen zu trennen, haben vorläufig nicht zum Ziele geführt. Die Arbeit in dieser Richtung wird aber fortgesetzt.

<sup>1)</sup> Arch. f. experim. Pathol. u. Pharmakol. 51, 240, 1904.

<sup>2)</sup> Ber. d. Deutsch. chem. Ges. 1909.

### Produkte der partiellen Hydrolyse mittels verdünnter Schwefelsäure.

Schmiedeberg und Alsberg<sup>1)</sup> ist es gelungen, bei der Hydrolyse mittels verdünnten Mineralsäuren ein phosphorhaltiges Spaltungsprodukt zu erhalten, welches sie Heminucleinsäure nannten. Ein analoges Produkt haben Osborn und Harris<sup>2)</sup> aus der Triticonucleinsäure erhalten. Und Kossel<sup>3)</sup> erwähnt, daß bei der Hydrolyse mittels verdünnter Mineralsäuren außer den Purinbasen noch eine phosphorhaltige Substanz entsteht. Vor einiger Zeit ist es Mandel und mir<sup>4)</sup> gelungen, bei der Hydrolyse der Thymonucleinsäure eine Substanz zu erhalten, welche die Zusammensetzung eines Komplexes Thymogluco-phosphorsäure besaß. Es wurde nun versucht, auf ähnliche Weise ein analoges Produkt aus der Hefenucleinsäure zu gewinnen. Es wurde dazu auf folgende Weise verfahren: 80,0 g der käuflichen Nucleinsäure wurden mit einem Liter 2%iger Schwefelsäure am Rückflußkühler 4 Stunden im Ölbad auf 125° C erhitzt. Beim Abkühlen schieden sich die Sulfate der Purinbasen aus. Zum Filtrat wird ein Überschuß von einer Lösung von Silbersulfat in verdünnter Schwefelsäure hinzugesetzt. Man bedarf etwa 100,0 g Silbersulfat. Es wurde über Nacht stehen gelassen, um eine möglichst vollständige Ausscheidung von Silberpurinen zu erhalten. Zum Filtrat von diesen fügt man eine konzentrierte Barytwasserlösung, bis zur alkalischen Reaktion. Der auf diese Weise entstandene Niederschlag wird in 2%iger Schwefelsäure aufgenommen, vom Silber mittels Schwefelwasserstoff befreit, dann mit Bariumcarbonat aufgeköcht und das Filtrat bei vermindertem Drucke zu ganz kleinem Volumen eingedampft. Die Lösung wurde mit Alkohol gefällt, der Niederschlag in Wasser gelöst und mit Alkohol umgefällt.

Die Substanz wurde im Vakuumexsiccator und dann bei vermindertem Druck über Phosphorpentoxyd bei 180° C getrocknet. Sie hatte die folgende Zusammensetzung:

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<sup>1)</sup> Arch. f. experim. Pathol. u. Pharmakol. 51, 240, 1904.

<sup>2)</sup> Zeitschr. f. physiol. Chem. 38, 113, 1402.

<sup>3)</sup> Arch. f. Anat. u. Physiol. 1891, 185.

<sup>4)</sup> Ber. d. Deutsch. chem. Ges. 41, 1905, 1908.

0,3210 g der Substanz gaben 0,2060 g  $\text{CO}_2$  und 0,0866 g  $\text{H}_2\text{O}$ ;  
 $\text{C} = 17,51\%$ ;  $\text{H} = 3,07\%$ .

0,3271 g der Substanz verbrauchten bei einer Stickstoffbestimmung nach Kjeldahl 0,110 cem  $\frac{2}{10}\text{-H}_2\text{SO}_4$ , mithin  
 $\text{N} = 4,70\%$ .

0,4819 g der Substanz gaben 0,2864 g  $\text{BaSO}_4$ ;  $\text{Ba} = 34,35\%$ .

0,5510 g der Substanz gaben 0,0814 g  $\text{Mg}_2\text{P}_2\text{O}_7$ ;  $\text{P} = 4,41\%$ .

Für das basische Bariumsals des Komplexes wurde verlangt:

C	H	N	P	Ba
17,64	1,96	4,57	5,06	44,70
17,57	3,07	4,70	4,41	

Die Substanz gab eine positive Orcinprobe, und bei der Destillation mittels Salzsäure vom spez. Gew. 1,06 lieferte sie Furfurol.

5,0 g dieser Substanz wurden in einem Einschmelzrohre 4 Stunden lang im Ölbad auf  $150^\circ\text{C}$  erhitzt. Die Flüssigkeit mit Äther extrahiert, die Schwefelsäure mit Barytwasser und der Überschuß an Baryt mittels Kohlensäure entfernt. Die eingedampften Filtrate wurden mit wässriger Pikrinsäurelösung behandelt, doch bildete sich kein Cytosinpikrat.

Die Pikrinsäure wurde darauf in der üblichen Weise entfernt und dann die Flüssigkeit mit einer Lösung von Silbernitrat und Barytwasser, behandelt. Es bildete sich ein Niederschlag, aus welchem man eine Substanz vom Aussehen des Uracils erhielt. Die Ausbeute reichte aber zur Analyse nicht aus. Aus dem ätherischen Auszuge ließ sich wieder eine Substanz mit einem Gehalt von  $53,30\%$  Ag darstellen.

Auf Grund der analytischen Zahlen kann man kaum die Substanz als einen einheitlichen Körper betrachten, doch ist das Verhältnis von  $\text{P}:\text{N}:\text{C}$  dem von einem Komplex, welcher aus Phosphorsäure, Pentose und Uracil besteht, ähnlich. Bei solch einem Komplex würde  $\text{P}:\text{N}:\text{C} = 1:0,9:3,9$  sein, bei der vorliegenden ist  $\text{P}:\text{N}:\text{C} = 1:1,06:3,7$ .

Die Resultate der Spaltung mittels  $25\%$ iger Schwefelsäure scheinen diese Ansicht über die Natur der Substanz zu stützen.

**310. P. A. Levene und W. A. Jacobs: Über die Pentose  
in den Nucleinsäuren.**

(Eingegangen am 14. April 1909.)

[Aus dem Rockefeller-Institute for Medical Research, New York.]

In den letzten Jahren ist nachgewiesen worden, daß einige Nucleinsäuren in ihrem Molekül eine Pentose enthalten. Eine eingehende Untersuchung über die Natur dieser Pentose wurde bisher nur von C. Neuberg<sup>1)</sup> ausgeführt. Dieser Forscher benutzte als Ausgangsmaterial das Nucleoprotein der Pankreasdrüse und kam zu dem Schluß, daß die darin vorliegende Pentose eine *l*-Xylose ist. Auch aus dem Nucleoprotein der Leber sollte man denselben Zucker isolieren können, und schließlich wollten Neuberg und Brahn<sup>2)</sup> die *l*-Xylose auch in der Inosinsäure nachgewiesen haben. Die Nucleinsäuren, in denen eine Pentose vorkommt, sind: die Guanylsäuren (aus der Leber und der Pankreasdrüse), die Hefe-Nucleinsäure und die Inosinsäure. Was die Natur der Pentose in der Inosinsäure angeht, so ist durch die Arbeiten von Haiser und Wenzel<sup>3)</sup> und von uns festgestellt, daß sie keine Xylose und auch

<sup>1)</sup> Diese Berichte 35, 1467 [1902].    <sup>2)</sup> Biochem. Ztschr. 5, 438 [1907].

<sup>3)</sup> Monatsh. für Chem. 30, 147 [1909].

keine Arabinose ist. Die Substanz ist auch keine Lyxose, da es uns gelungen ist, aus synthetisch dargestellter Lyxose ein Xylosazon zu gewinnen, während das Osazon der Carnose (wir möchten provisorisch diesen Namen für die Pentose vorschlagen) ein Osazon mit anderen Eigenschaften liefert. Auch einige Derivate der Carnose sind von denen der Ribose wie sie von Fischer und Piloty angegeben sind, in ihren Eigenschaften abweichend. Die schon jetzt erhaltenen Resultate scheinen jedoch zu der Annahme zu berechtigen, daß die Carnose zur Arabinose-Gruppe gehört.

Nun hat sich erwiesen, daß die Pentose in anderen Nucleinsäuren der Carnose in manchen Eigenschaften ähnlich ist, und daß sie sich von der *l*-Xylose unterscheidet. Schon vor einem Jahre haben Levene und Mandel<sup>1)</sup> die Beobachtung gemacht, daß man bei der Hydrolyse der Leber-Guanylsäure zu einer linksdrehenden Pentose gelangt. Das Drehungsvermögen des Osazons konnte aber wegen der Dunkelheit der Lösung nicht mit Sicherheit bestimmt werden. Nun ist es uns jetzt gelungen, durch Umkrystallisieren aus Pyridin-Wasser-Gemisch Osazone zu erhalten, welche sich in Pyridin-Alkohol nach Neuberg mit ganz heller Farbe auflösten, und so ist es möglich geworden, festzustellen, daß das Osazon ein anderes Drehungsvermögen als die Xylose besitzt.

Weiter ist es gelungen, zu bestimmen, daß das Drehungsvermögen des Zuckers aus der Leber-Guanylsäure denselben Wert besitzt wie bei der Carnose.

Auch aus der Pankreas-Guanylsäure gelang es, einen linksdrehenden Zucker zu erhalten, welcher ein Phenylosazon mit den Eigenschaften des Carnosazons lieferte.

Aus der Hefe-Nucleinsäure ist es ebenfalls gelungen, ein Phenylosazon mit demselben Drehungsvermögen wie das Carnosazon zu erhalten. Es ist uns aber bis jetzt nicht gelungen, den freien Zucker in genügender Reinheit darzustellen, um sein wahres Drehungsvermögen zu bestimmen. Dieses ist aber Boos<sup>2)</sup> gelungen. Bei der Hydrolyse des Kupfersalzes der Hefe-Nucleinsäure erhielt er eine linksdrehende Lösung, aus welcher er weiter ein Phenylosazon und ein Benzyl-phenyl-hydrazon darstellte. Scheinbar waren die von ihm erhaltenen Derivate stark verunreinigt, da sie ihn zu kaum möglichen Schlüssen über die Natur der reduzierenden Substanz führten. Auffallend ist, daß der Schmelzpunkt des Benzyl-phenyl-hydrazons, welches Boos darstellte, mit dem Schmelzpunkte des rohen Benzyl-phenyl-hydrazons der Carnose identisch war. Beim Umkrystallisieren aus

<sup>1)</sup> Biochem. Ztschr. 10, 221 [1908].

<sup>2)</sup> Journ. f. Biol. Chemistry 5, 469 [1908].

Essigäther stieg der Schmelzpunkt des Carnose-Benzyl-phenyl-hydrazons aber an.

Nach alledem muß die alte Ansicht über die Natur der in Nucleinsäuren vorkommenden Pentose aufgegeben werden. Mit der Aufklärung der wahren Natur der Substanz sind wir jetzt beschäftigt.

### Experimenteller Teil.

Die Inosinsäure. — In früheren Mitteilungen über die Inosinsäure wurde schon angegeben, daß die aus dieser Substanz erhaltene Pentose sich von der *l*-Xylose und *l*-Arabinose unterscheidet, und zwar in Folgendem: Im Schmelzpunkt und Drehungsvermögen des Zuckers, im Schmelzpunkte des Benzyl-phenyl-hydrazons. Seitdem sind noch andere Derivate dargestellt worden, welche sich von den entsprechenden Derivaten der *l*-Xylose oder *l*-Arabinose unterscheiden. Das Vorliegen von *d*-Lyxose muß man gleichfalls auf Grund des Drehungsvermögens des Phenylsazons ausschließen.

Phenylhydrazon. 0.4 g des Zuckers wurden in möglichst wenig Alkohol gelöst und mit 0.27 g Phenylhydrazin versetzt. Die Lösung wurde bei 35° 24 Stunden lang stehen gelassen. Dann krystallisierten große, dicke, sechseckige Prismen aus. Die Lösung wurde mit mehreren Volumen trocknen Äthers versetzt und im Eisschrank 18 Stunden lang stehen gelassen. Hierdurch schieden sich noch lange Nadeln aus. Die Krystalle wurden abgesaugt und zweimal aus wenig Alkohol umkrystallisiert. Der Schmelzpunkt blieb konstant. Aus der Mutterlauge konnte durch Petroläther noch mehr Substanz gewonnen werden. Der Körper aus der alkoholischen Lösung bildet lange, seidenartige Nadeln; im Capillarrohr rasch erhitzt, schmilzt er zwischen 124° und 127°. Die Arabinose- und Riboseverbindungen schmelzen bei 153° resp. 154—155°. Im Wasser ist er besonders beim Erwärmen löslich. Leicht löslich in heißem Alkohol, Aceton, Essigäther, schwer in den kalten Lösungsmitteln, unlöslich in Äther und Petroläther.

0.1507 g Sbst. wurden in 4 ccm absolutem Alkohol gelöst; Gesamtgewicht der Lösung 3.7897 g; drehte im 0.5-dm-Rohr mit Natriumlicht 0.09° nach rechts. Mithin  $[\alpha]_D^{20} = +4.53^\circ$ .

*p*-Bromphenylhydrazon. Dies Derivat wird in derselben Weise dargestellt wie oben beim Phenylhydrazon beschrieben. Die alkoholische Lösung muß aber mit trockenem Äther versetzt werden, bevor die Krystallisation anfängt. Dabei scheiden sich farblose, seidenartige Nadeln aus. Nach 24-stündigem Stehen im Eisschrank werden die Krystalle abfiltriert und zweimal aus wenig absolutem Alkohol umkrystallisiert. — Rasch erhitzt, fängt der Körper gegen 168° an zu sintern, und bei 172—173° (korr.) schmilzt er unter langsamer Zersetzung. Die Arabinose-, Xylose-

und Ribose-Verbindungen schmelzen bei 160° resp. 128° und 165°. In kaltem Wasser und Alkohol löst sich der Körper kaum, aber leicht in heißem Wasser und heißem Alkohol, der ihn allmählich aufnimmt. In anderen Lösungsmitteln gleicht er dem Phenylhydrazon.

0.2025 g Subst. werden in 5 ccm absolutem Alkohol gelöst; Gesamtgewicht der Lösung 4.4280 g; drehte mit Natriumlicht im 0.5-dm-Rohr 0.13° nach links. Mithin  $[\alpha]_D^{20} = -5.69^\circ$ .

Die Leber-Guanylsäure. — 2 g der Substanz wurden mit 100 ccm 4-prozentiger Schwefelsäure übergossen und über Nacht stehen gelassen und dadurch eine vollständige Lösung der Substanz erreicht. Die Lösung wurde dann am Rückflußkühler 4 Stunden lang erhitzt. Die ursprünglich rechtsdrehende Lösung erwies sich dann als linksdrehend; die Hydrolyse wurde an diesem Punkt unterbrochen. Die Purinbasen entfernte man in üblicher Weise mittels Silbersulfat; das Filtrat vom Purinsilber wurde von Silber und Schwefelsäure befreit und bei vermindertem Druck eingedampft. Der Rückstand wurde mit heißem 98-prozentigem Alkohol ausgezogen, der Alkohol abgedampft und der Rückstand in 25 ccm Wasser gelöst. Diese Lösung wurde dann auf das Drehungsvermögen und auf die Reduktionskraft für Fehlingsche Lösung untersucht. Die Reduktionskraft entsprach einem Pentose-Gehalt von etwa 0.075 g; das Drehungsvermögen war im 2-dm-Rohre  $-0.12^\circ$ .

Berechnet man das Drehungsvermögen der Carnose in der oben angegebenen Konzentration, so würde man dasselbe Drehungsvermögen erhalten. In dieser Hinsicht scheinen die Pentosen der beiden Nucleinsäuren einander ähnlich zu sein.

Die Zuckerlösung wurde dann zur Darstellung des Phenylsazons verbraucht. Auch hier erhielt man Präparate, welche mit heller Farbe in Lösung gingen, wenn man das Umkrystallisieren aus einem Pyridin-Wasser-Gemisch durchführte. Das Osazon hatte den Schmelzpunkt 163°.

0.050 g der Substanz, in 5 ccm Pyridin-Alkohol (nach Neuberg) gelöst, drehten im 0.5 dm-Rohr im Natriumlicht  $-0.23^\circ$ , besaßen also dasselbe Drehungsvermögen wie das Carnosazon und das Arabinosazon.

Die Pankreas-Guanylsäure. — 1 g Guanylsäure (biuretfrei, aber sonst nicht absolut rein) wurde in 100 ccm 2-prozentiger Schwefelsäure 8 Stunden am Rückflußkühler gekocht. Das ursprüngliche Drehungsvermögen der Lösung betrug bei Natriumlicht und im 0.865-dm-Rohr  $+0.07^\circ$ . Am Ende des Experimentes drehte dieselbe Lösung  $-0.05^\circ$ . Nach dem Entfernen der Purinbasen erhielt man 13 ccm einer Lösung, welche unter denselben Bedingungen das Drehungsvermögen von  $-0.25^\circ$  besaß. Aus dieser Lösung erhielt man ein Phenylsazon vom Schmp. 163°.

0.050 g der Substanz, in 5 ccm Pyridin-Alkohol gelöst, besaßen im 0.5-dm-Rohr das Drehungsvermögen von  $-0.23^{\circ}$ .

Also auch diese Pentose schien der Carnose ähnlich zu sein.

Die Hefe-Nuclainsäure. — 80 g der käuflichen Nucleinsäure wurden mit 1 Liter 2-prozentiger Schwefelsäure am Rückflußkühler im Ölbad 4 Stunden lang gekocht. Die Purinbasen wurden mittels Silbersulfat entfernt. Aus dem Filtrat wurden die Zwischenprodukte der Hydrolyse mittels Silber- und Barytlösung entfernt und das Filtrat dieses Niederschlages von Silber und Barium befreit. Die so erhaltene Lösung wurde auf 220 ccm eingedampft. 1.5 ccm reduzierten 0.0874 g Kupfer, enthielten also etwa 0.052 g Zucker. Die totale Ausbeute an Zucker betrug etwa 7.5 g.

80 ccm der ursprünglichen Lösung wurden mit einer Lösung von 10 g Phenylhydrazin in Eisessig auf dem Wasserbade erhitzt. Das Osazon wurde aus wenig Wasser, welches pyridinhaltig war, umkrystallisiert und zur Analyse gebracht. Es wurde zuerst im Vakuum-exsiccator über Schwefelsäure und dann bei  $108^{\circ}$  unter vermindertem Druck über Phosphorpentoxyd getrocknet. Schmp.  $162^{\circ}$ .

0.0688 g Sbst.: 0.1578 g  $\text{CO}_2$ , 0.0398 g  $\text{H}_2\text{O}$ .

$\text{C}_{17}\text{H}_{20}\text{N}_4\text{O}_3$ . Ber. C 62.20, H 6.09.

Gef. » 62.55, » 6.47.

0.2000 g Sbst. wurden in einem Gemisch von 6 ccm Alkohol und 4 g Pyridin gelöst. Im 0.5-dm-Rohr drehte die Lösung  $-0.42^{\circ}$ .



**476. P. A. Levene und W. A. Jacobs: Über die Pentose  
in den Nucleinsäuren.**

(II. Mitteilung.)

[Aus dem Rockefeller Institute for Medical Research, New York.]

(Eingegangen am 16. Juli 1909.)

Bei der Besprechung der Natur der Pentose des Inosins und damit auch der der anderen Nucleinsäuren haben wir<sup>1)</sup> die Ansicht ausgedrückt, daß die Substanz *d*-Ribose sein könnte und zwar aus folgenden Gründen. Nach der Furfurol-Ausbeute mußte die Substanz als eine Aldose anerkannt werden. Nun sind nach der Theorie nur vier optisch-aktive Aldopentosen und ihre Antipoden möglich. Von diesen waren bisher nur drei, nämlich Arabinose, Xylose und Lyxose, in krystallinischer Form bekannt. Das Drehungsvermögen und die Schmelzpunkte dieser Substanzen waren genau studiert. Die *l*-Arabinose, *l*-Xylose und *l*-Lyxose drehten nach rechts, deren Antipoden nach links. Der Inosinsäure-Zucker ist linksdrehend und konnte deshalb nur zur zweiten Gruppe gehören. Betrachtet man aber das Drehvermögen des Zuckers und des aus dem Zucker erhaltenen Phenylsazons, so konnte man das Vorliegen einer Xylose oder einer Lyxose (wie es Haiser und Wenzel annehmen)<sup>2)</sup> ausschließen. Von

<sup>1)</sup> Diese Berichte **42**, 2102 [1909]. <sup>2)</sup> Monatsh. f. Chem. **30**, 377 [1909].

*d*-Arabinose unterschieden sich im Schmelzpunkt und Drehungsvermögen (des freien Zuckers), und auch im Verhalten des Benzylphenyl-, Phenyl- und *p*-Bromphenylhydrazons. Nach diesen Überlegungen mußte man das Vorliegen der *d*-Ribose als wahrscheinlich annehmen. Diesen Gedanken haben wir mit großer Vorsicht ausgesprochen, hauptsächlich weil es uns zu jener Zeit, aus Mangel an Material nicht gelang, die Ribonsäure oder die Ribotrioxylglutarsäure darzustellen. Ferner harmonisierten unsere Befunde über den Schmelzpunkt des Phenylhydrazons mit dem von Fischer und Piloty<sup>1)</sup> für die Ribose angegebenen nicht; und die Eigenschaften des *p*-Bromphenylosazons waren mit den Angaben von Neuberg<sup>2)</sup> über das entsprechende Osazon der Arabinose im Widerspruch, während sie nach der Theorie identisch sein mußten.

Nun aber scheint uns jetzt die Natur des Zuckers als *d*-Ribose bewiesen, und zwar aus folgenden Gründen: Bald nach der Veröffentlichung unserer ersten Mitteilung erhielten wir eine Privatmitteilung, daß es A. van Ekenstein und Blankisma gelungen sei, die krystallinische *l*-Ribose synthetisch darzustellen. Der Schmelzpunkt dieses Zuckers ist 87°, ebenso hoch wie der unseres Zuckers, ferner war das Drehungsvermögen der *l*-Ribose  $[\alpha]_D = +18.8^\circ$ , des unseren  $[\alpha]_D = +19.3^\circ$ . van Ekenstein und Blankisma hofften, durch weitere Reinigung der Substanz das Drehungsvermögen steigern zu können. Sie waren nur im Besitze von wenig Material. Die Resultate der Arbeit von van Ekenstein und Blankisma sind jetzt veröffentlicht worden<sup>3)</sup>. Ferner gelang es uns, aus dem Zucker das Lacton einer inaktiven Trioxylglutarsäure mit dem Schmelzpunkt des Ribotrioxylglutarsäurelactons zu erhalten. Endlich gelang es uns zu beweisen, daß das *p*-Bromphenylosazon unsres Zuckers und das der *l*-Arabinose Antipoden sind, daß aber die Angaben Neubergs über das Arabinose-Derivat noch nicht ganz richtig waren.

Da die Pentose der Guanylsäure und der Hefenucleinsäure mit der der Inosinsäure identisch sind, so darf man jetzt die Natur der Pentose auch in diesen Nucleinsäuren als *d*-Ribose betrachten.

### Experimenteller Teil.

#### *d*-Ribo-trioxyglutarsäurelacton.

5 g der Pentose, aus Karnin dargestellt, wurden nach der Kilian-schen Methode in 2½ Teilen Salpetersäure vom sp. Gew. 1.2 acht Stunden bei 40° gehalten und dann am Wasserbade in einer Platin-

<sup>1)</sup> Diese Berichte 24, 4214 [1891].    <sup>2)</sup> Diese Berichte 32, 3384 [1899].

<sup>3)</sup> Chemisch Weekblad 1902, Nr. 22.

schale unter stetem Umrühren rasch eingedampft. Nach Zugabe von wenig Wasser und abermaligem Eindampfen wurden die flüchtigen Produkte möglichst vertrieben. Der Sirup wurde in 125 ccm Wasser aufgenommen und mit frisch bereitetem Calciumcarbonat gekocht, bis die Lösung neutral reagierte. Im Filtrat schied sich beim Stehen im Eisschrank nur eine kleine Menge rotgefärbtes Calciumsalz aus. Nach Einengen des Filtrats war die Ausbeute auch nur sehr gering; das Salz wurde wahrscheinlich von Verunreinigungen in Lösung gehalten. Zur Trennung wurde die Lösung sorgfältig mittels Alkohol fraktioniert; so ist es uns gelungen, das Calciumsalz zu erhalten. Das Salz löste sich nicht in Wasser, beim Kochen quoll es auf.

2 g des Salzes wurden in Wasser suspendiert, auf dem Wasserbade erhitzt und mit genau der berechneten Menge Oxalsäure zerlegt. Die Lösung wurde mit Tierkohle entfärbt und auf dem Wasserbade konzentriert. Der Sirup wurde in das Vakuum über Schwefelsäure gestellt, wobei bald die Krystallisation anfang. Beim Umrühren erstarrte das Ganze. Die Substanz wurde mit viel trockenem Essigäther ausgekocht und die vereinigten Lösungen stark eingengt. Es schieden sich am Rande des Gefäßes kleine weiße Warzen, aus mikroskopischen Nadeln bestehend, aus, genau wie Fischer und Piloty sie beschrieben haben. Der Körper wurde noch zweimal umkrystallisiert und im Vakuum getrocknet.

Im Capillarrohr erhitzt, fing er gegen  $158^{\circ}$  an zu erweichen, und gegen  $168^{\circ}$  schmolz er unter Gasentwicklung. Von Fischer und Piloty wurde der Schmelzpunkt zu  $160\text{--}170^{\circ}$  angegeben, aber bei Körpern von solch unscharfen Schmelzpunkten sind kleine Unterschiede wohl erklärlich. Eine ungefähr 5-prozentige Lösung zeigte keine merkliche Drehung, möglicher Versuchsfehler  $0.02^{\circ}$ . Zur Analyse wurde die Substanz im Vakuum über Schwefelsäure getrocknet.

0.1003 g Subst.: 0.1362 g  $\text{CO}_2$ , 0.0392 g  $\text{H}_2\text{O}$ .

$\text{C}_5\text{H}_6\text{O}_6$ . Ber. C 37.04, H 3.70.

Gef. » 37.03, » 4.34.

#### *p*-Bromphenyllosazon der *d*-Ribose.

Da nach der Theorie das Osazon der *d*-Ribose identisch mit dem der Arabinose sein sollte, haben wir die beiden Substanzen zum Vergleich dargestellt. Sie haben sich mit Ausnahme der Drehungsrichtung als identisch erwiesen. Die Eigenschaften unseres Präparates wichen aber in manchen Hinsichten von den Angaben Neubergs ab.

1 g *d*-Ribose wurde in 75 ccm Wasser aufgelöst und mit 3.7 g *p*-Bromphenylhydrazin, in 10 ccm Eisessig gelöst, versetzt. Die Mischung wurde anderthalb Stunden auf dem Wasserbade erhitzt. Wäh-

rend der Operation schieden sich dunkel gefärbte Plättchen ab, die mit viel Öl vermischt waren. Beim Stehen im Eisschrank erstarrte das Öl ebenfalls. Die Krystalle wurden abgesaugt und gewaschen. Das Produkt wurde in 50 ccm Alkohol gelöst, mit einigen ccm Eisessig versetzt und mit Tierkohle erhitzt. Das warme Filtrat wurde mit heißem Wasser bis zur Trübung versetzt und erkalten gelassen. Der Niederschlag, der noch dunkel gefärbt war, bestand aus undeutlich krystallinischen Flocken und Öltröpfchen. Die Reindarstellung der Substanz ist ziemlich schwierig. Man verfährt daher am besten, indem man den Körper wiederholt aus Alkohol mittels heißem Wasser fällt, bis aus der Emulsion der krystallinische Teil sich aus der warmen Lösung rasch am Boden des Gefäßes absetzt. Die noch warme, getrübbte Mutterlauge wird möglichst abdekantiert und dann filtriert; die hellgelben Krystalle werden dann gleichfalls mehrmals aus Alkohol gefällt. Endlich bekommt man den Körper rein in hellgelben, glänzenden Plättchen, die unter dem Mikroskop als wohlausgebildete, sechseckige Platten erscheinen.

Im Capillarrohr rasch erhitzt, sintert er gegen  $175^{\circ}$  und schmilzt beim weiteren Erhitzen zwischen  $180$ — $185^{\circ}$  (korr.). Es ist uns nie gelungen, den Neubergschen Schmp.  $196$ — $200^{\circ}$  zu erhalten. In heißem Wasser ist der Körper kaum löslich, leicht aber in Alkohol, Äther und Pyridin. Das optische Verhalten war auch ein anderes, als Neuberg angibt. In Alkohol und in der Neubergschen Alkohol-Pyridin-Mischung zeigte die Substanz Birotation.

0.1004 g Sbst. in 5 ccm Pyridin-Alkohol-Mischung (2:3) gelöst. Die Drehung war innerhalb 10 Minuten nach dem Auflösen im 0.5-dm-Rohr mit Natriumlicht  $-0.56^{\circ}$ . Nach 15 Minuten war sie  $-0.52^{\circ}$ . Die Drehung nahm dann regelmäßig ab. Nach 24 Stunden blieb sie bei  $-0.36^{\circ}$  konstant.

0.0499 g Sbst. wurden in 5 ccm Alkohol gelöst. Innerhalb 10 Minuten nach dem Auflösen betrug die Drehung im 0.5-dm-Rohr mit Natriumlicht  $0.12^{\circ}$  nach links, nach 24 Stunden blieb sie bei  $-0.08^{\circ}$  konstant.

Zur Analyse wurde im Vakuum über Schwefelsäure getrocknet.

0.1515 g Sbst.: 16.7 ccm N ( $30.5^{\circ}$ , 754 mm).

$C_{17}H_{18}O_2N_4Br_2$ . Ber. N 11.52. Gef. N 11.82.

Die Substanz löst sich leicht in Pyridin. Wenn aber die warme Lösung mit heißem Wasser bis zur Trübung versetzt wird, bildet sich beim Erkalten eine Emulsion von Öltröpfchen, die nach einiger Zeit zu einem dicken Brei von langen, dünnen, verfilzten Nadeln erstarren. Nach dem Absaugen und Trocknen im Vakuum über Schwefelsäure riecht der Körper stark nach Pyridin. Beim längeren Stehen über Schwefelsäure verschwindet der Geruch nicht. Die Substanz ist anscheinend eine Pyridinverbindung.

Im Capillarrohr erhitzt, sintert sie bei 75°, und bei 80—85° schrumpft sie zusammen, wahrscheinlich unter Abgabe von Pyridin. Im Vakuum über Phosphorsäureanhydrid bei 60° erhitzt, verliert der Körper nur wenig am Gewicht. An feuchter Luft aber gibt er langsam das Pyridin ab.

0.1230 g Sbst. wurden im Vakuumexsiccator über 5-prozentiger Schwefelsäure 24 Stunden stehen gelassen. Der Geruch nach Pyridin war vollständig verschwunden. Der Körper wurde dann weiter im Vakuum über Phosphorsäureanhydrid zur Gewichtskonstanz erhitzt. Er wog dann 0.1099 g. Das gebundene Pyridin betrug also 0.131 g oder 10.65 %. Für 1 Mol. Pyridin sind 14 % berechnet. Der niedrigere Wert ist dadurch zu erklären, daß beim ursprünglichen Trocknen der Substanz etwas vom gebundenen Pyridin weggetrieben sein konnte.

0.1100 g pyridinfreie Sbst.: 11.4 ccm N (29°, 760.5 mm).

$C_{17}H_{18}O_3N_4Br_2$ . Ber. N 11.52. Gef. N 11.77.

Die auf diese Weise vom Pyridin befreite Substanz schmilzt gegen 170°.

#### *l*-Arabinose-*p*-bromphenylosazon.

Das Derivat der *l*-Arabinose wurde in genau derselben Weise dargestellt, wie oben angegeben. In Schmelzpunkt, Krystallform und optischem Verhalten war es genau wie bei den Antipoden angegeben.

0.1369 g Sbst.: 14.9 ccm N (29°, 755 mm).

$C_{17}H_{18}O_3N_4Br_2$ . Ber. N 11.52. Gef. N 11.60.

0.1 g Sbst., in 5 ccm Pyridin-Alkohol-Mischung gelöst, zeigte innerhalb 10 Minuten nach dem Auflösen im 0.5-dcm-Rohr mit Natriumlicht die Drehung + 0.56°. Nach 24 Stunden war sie auf + 0.40° gesunken, was innerhalb des Versuchsfehlers mit gefärbten Lösungen mit der bei den Antipoden angegebenen Drehung übereinstimmt.

0.0501 g Sbst., in 5 ccm Alkohol gelöst, zeigte im 0.5-dcm-Rohr mit Natriumlicht innerhalb 10 Minuten nach dem Auflösen die Drehung + 0.13°. Nach 24 Stunden betrug sie + 0.09°.

Die Pyridinverbindung wurde auch hier dargestellt. 0.1394 g, wie oben behandelt, verloren 0.0157 g Pyridin oder 11.26 %. Für 1 Mol. sind 14 % berechnet.

0.1230 g pyridinfreie Sbst.: 12.8 ccm N (29°, 756 mm).

$C_{17}H_{18}O_3N_4Br_2$ . Ber. N 11.52. Gef. N 11.75.

Furfurolbestimmung. 0.1995 g der Zucker wurden nach der Methode von Tollens und Grund mittels Salzsäure (spez. Gew. 1.06) destilliert und das Phloroglucid gewogen. Es betrug 0.1856 g, wie für eine Aldose berechnet ist.



### 360. P. A. Levene und W. A. Jacobs: Über Guanylsäure

(I. Mitteilung.)

[Aus dem Rockefeller-Institute for Medical Research, New York.]

(Eingegangen am 19. Mai 1909.)

Als »Guanylsäure« ist im Jahre<sup>1)</sup> 1899 von Bang eine Substanz beschrieben worden, die nach einem Verfahren von Hammarsten aus der Pankreasdrüse dargestellt war und alle Eigenschaften einer Nucleinsäure besaß. Nach Bang sollten als Komponenten dieser Substanz Phosphorsäure, Glycerin, eine Pentose und Guanin fungieren; in Bezug auf die genauere Konstitution der Säure enthält seine Arbeit jedoch keine experimentell begründeten Annahmen. Neuberg<sup>2)</sup> erweiterte diese Angaben von Bang über die Eigenschaften der Komponenten, indem er die Pentose als *l*-Xylose ansprach. In den letzten Jahren beschäftigten sich v. Fürth und Jerusalem<sup>3)</sup> mit der Untersuchung der Substanz und erklärten, daß Glycerin unter den Spaltungsprodukten der Guanylsäure nicht vorkomme. Diese Behauptung wurde dann von Steudel<sup>4)</sup> bestätigt. Jones<sup>5)</sup> entdeckte die Guanylsäure auch in der Milz, während Levene und Mandel<sup>6)</sup> sie in der Leber auffanden. Die letzteren Forscher haben unsere Kenntnisse über die Natur der Säure insoweit erweitert, als sie das optische Drehungsvermögen derselben beschrieben und beobachteten, daß die bei der Hydrolyse der Guanylsäure resultierende Zuckerslösung nicht rechtsdrehend (wie dies die *l*-Xylose verlangen würde), sondern linksdrehend war.

Sichere Angaben über die Konstitution der Guanylsäure lagen mithin bisher noch nicht vor.

Nun sind aber während des letzten Jahres unsere Kenntnisse über die Konstitution anderer Nucleinsäuren wesentlich erweitert worden. Levene und Mandel<sup>7)</sup> haben die Beobachtung gemacht, daß bei der partiellen Spaltung der Thymonucleinsäure, außer Phosphorsäure und Basen, Körper entstehen, die nur aus Kohlehydrat und Base zusammengesetzt sind, und ferner Substanzen, die aus Phosphorsäure, Hexose und Base in äquimolekularem Verhältnis bestehen. Sie nannten diese Substanzen Nucleotide. Später haben

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<sup>1)</sup> Ztschr. für physiolog. Chem. **26**, 133; **31**, 411.

<sup>2)</sup> Diese Berichte **32**, 3386 [1899].

<sup>3)</sup> Beitr. zur chem. Physiolog. u. Path. **10/11**, 146 [1908].

<sup>4)</sup> Ztschr. für physiolog. Chem. **53**, 530 [1908].

<sup>5)</sup> Journ. of biolog. Chem. **4**, 289 [1908].

<sup>6)</sup> Biochem. Ztschr. **10**, 221 [1908]. <sup>7)</sup> Diese Berichte **41**, 1905 [1908].

Levene und Jacobs<sup>1)</sup> bei der Hydrolyse der Inosinsäure die Bildung einer Pentose-phosphorsäure und eines Komplexes Pentose-hypoxanthin erwiesen. In einer weiteren Arbeit<sup>2)</sup> haben sie die letzterwähnte Substanz als das Inosin, welches Haiser und Wenzel<sup>3)</sup> aus Carnin dargestellt hatten, erkannt. Es ist also wenigstens für eine Nucleinsäure die Anordnung der einzelnen Komponenten aufgeklärt worden.

Es ist uns nun jetzt gelungen, zu beweisen, daß auch die »Guanylsäure« der Inosinsäure in ihrer Konstitution ähnlich ist. Wir erhielten nämlich aus ihr eine schön krystallisierte Substanz von der Zusammensetzung  $C_{10}H_{13}N_5O_5$ , die sich aus ihren Lösungen in langen Nadeln vom Aussehen des Tyrosins abschied. Sie besaß den Schmp.  $237^{\circ}$  und — in 1 Mol. Natronlauge gelöst — das Drehungsvermögen von  $-60.52^{\circ}$ . Bei der Hydrolyse mittels verdünnter Mineralsäure bildeten sich eine Pentose und Guanin. Gegen Erhitzen mit Alkalilösungen ist die Substanz dagegen sehr resistent. Sie reduziert Fehlingsche Lösung nicht, wohl aber nach vorheriger Hydrolyse mittels Säuren. Die Substanz enthält also Pentose und Guanin in glykosidartiger Form gebunden. Wir wollen das Guanin-pentosid der Kürze halber Guanosin nennen. Die Existenz dieser glykosidartigen Substanz bringt den Beweis, daß in der Guanylsäure die Purinbase an die Pentose gebunden ist. Wir halten uns ferner für berechtigt, anzunehmen, daß die Pentose an die Phosphorsäure gebunden ist, obwohl wir gegenwärtig die Pentose-phosphorsäure noch nicht isoliert haben. Daß diese Substanz aber bei der Hydrolyse entsteht, schließen wir aus den folgenden Gründen: Die Pentose und das Guanosin sind linksdrehend. Bei der Hydrolyse der Guanylsäure gelangt man zu einer Phase, in der das Guanin fast vollkommen abgespalten, die Lösung aber noch rechtsdrehend ist, um dann bei weiterer Spaltung linksdrehend zu werden. Die Rechtsdrehung kann also durch die Bildung von Pentose-phosphorsäure erklärt werden. Wir hoffen indessen, die Substanz noch als solche in reiner Form isolieren zu können.

Wir wollen hier noch erwähnen, daß, während Guanosin ebenso wie Inosin linksdrehend ist, die Guanylsäure ein der Inosinsäure entgegengesetztes Drehungsvermögen besitzt. Diese Tatsache könnte durch die Annahme erklärt werden, daß in den entsprechenden Nucleinsäuren die Phosphorsäure an verschiedene Hydroxylgruppen der Pentose gebunden ist.

<sup>1)</sup> Diese Berichte **41**, 2703 [1908].    <sup>2)</sup> Diese Berichte **42**, 335 [1909].

<sup>3)</sup> Monatsh. für Chem. **29**, 157 [1908].

Über die Natur der Pentose<sup>1)</sup> wollen wir weiter berichten, daß sie mit der aus Inosin erhaltenen Carnose identisch ist; sie ließ sich in krystallinischer Form erhalten, zeigte denselben Schmelzpunkt und das gleiche Drehungsvermögen und gab auch dasselbe Osazon. Es gelingt, die reine Pentose aus dem Guanosin, wie auch aus der Guanylsäure direkt zu erhalten, doch gewinnt man sie viel leichter aus der ersten Substanz. Die Ansicht von Neuberg, daß die Pentose der Guanylsäure eine *l*-Xylose sei, muß nunmehr aufgegeben werden.

Ferner wollen wir hier noch erwähnen, daß der eine von uns die Bildung von ähnlichen glykosidähnlichen Körpern auch bei der alkalischen Hydrolyse der Hefe-nucleinsäure beobachtet hat, und daß wir mit der Isolierung dieser Substanz, wie auch mit ihrer synthetischen Darstellung beschäftigt sind.

### Experimenteller Teil.

#### Die Säure-Hydrolyse der Guanylsäure.

10 g Guanylsäure, die direkt aus den Pankreasdrüsen nach einer Methode, die in einer späteren Mitteilung veröffentlicht werden soll, dargestellt wurde, haben wir 8 Stunden in 500 ccm 2-prozentiger Schwefelsäure am Rückflußkühler gekocht. Die Lösung zeigte keine optische Aktivität. Nach Zugabe von 1 % Salzsäure wurde dann das Kochen noch 4 Stunden lang fortgesetzt. Die Lösung erwies sich auch jetzt noch als inaktiv, zeigte aber intensiv reduzierende Eigenschaften und gab stark die Orcinprobe. Organisch gebundener Phosphor war noch zugegen, da aber das Guanin vollständig abgespalten war, wurde die Hydrolyse nunmehr unterbrochen. Die Lösung wurde mittels Silbersulfat von der Purinbase befreit und das Filtrat zuerst mit Schwefelwasserstoff behandelt und dann mit Bariumcarbonat gekocht, bis die Schwefelsäure entfernt war. Das Filtrat wurde unter vermindertem Druck zur Trockne eingedampft und der Rückstand in wenig Wasser gelöst. Das Filtrat zeigte deutlich alkalische Reaktion, was von der Anwesenheit beträchtlicher Mengen anorganischer Materie im Ausgangsmaterial herrührte. Die Lösung wurde mit Schwefelsäure schwach sauer gemacht und mit viel absolutem Alkohol gefällt. Das Filtrat wurde dann wieder zur Trockne verdampft und von neuem in Wasser gelöst. Die Schwefelsäure wurde wiederum durch überschüssiges Bariumcarbonat entfernt und das Filtrat zur Trockne eingedampft. Der sirupartige Rückstand wurde dann in absolutem Alkohol aufgenommen und ein halbes Volumen Äther hinzugesetzt. Nach dem Stehen im Eisschrank wurde das Filtrat im Vakuum eingedampft und

<sup>1)</sup> Vgl. hierüber die nächstfolgende Mitteilung (S. 2476) derselben Autoren, welche bei der Redaktion später eingegangen ist.

der Rückstand in 50 ccm Wasser gelöst. Das Rotationsvermögen, auf Carnose berechnet, wie auch die Reduktionskraft zeigten einen Gehalt von 1.0 g Zucker an.

Die wäßrige Lösung wurde wieder zur Trockne verdampft und der Rückstand in wenig absolutem Alkohol aufgenommen. Dann wurden 1.2 g *p*-Bromphenylhydrazin, in wenig Alkohol gelöst, hinzugefügt und die Lösung im Exsiccator über Schwefelsäure über Nacht stehen gelassen. Hierbei schieden sich weiße, kuglige Aggregate aus. Die Krystalle wurden abgesaugt und zweimal aus wenig heißem Wasser umkrystallisiert. Die Substanz erwies sich dann im Schmelzpunkt und in der optischen Aktivität als mit dem Carnose-*p*-bromphenylhydrazon identisch.

0.1182 g Subst.: 9.8 ccm N (über 50-prozentiger Kalilauge) (23°, 756 mm).

$C_{11}H_{15}BrN_2O_4$ . Ber. N 8.78. Gef. N 9.28.

### Neutrale Hydrolyse.

2.5 g Guanylsäure wurden in einem kleinen Überschuß von normaler Natronlauge unter Erwärmen aufgelöst. Die Lösung wurde dann mit Essigsäure versetzt, bis sie gegen Lackmus neutral reagierte, hiernach auf 75 ccm verdünnt und im Einschlußrohr auf 130–135° erhitzt. Beim Erkalten erstarrte der Inhalt des Rohrs zu einer gelatinösen Masse, die sich unter dem Mikroskop in sehr dünne, verfilzte Nadeln auflöste. Die Mischung wurde einige Zeit in Eis gestellt, dann abgesaugt und die Substanz in wenig heißem Wasser unter Zusatz von Tierkohle aufgelöst. Das Filtrat erstarrte beim Erkalten zu einem Brei von langen, dünnen, seidenartigen, tyrosinähnlichen Nadeln. Sie wurden abermals aus Wasser umgelöst und hiernach an der Luft bis zum konstanten Gewicht stehen gelassen. Die Ausbeute an ganz reinem Produkt betrug 0.75 g.

Die Substanz enthielt keinen gebundenen Phosphor und gab sehr stark die Pentose-Reaktion. Beim Kochen mit Säuren wurde sie leicht gespalten unter Auftreten reduzierender Eigenschaften und Bildung von freiem Guanin. Der Körper ist also eine Verbindung von Guanin und Carnose, die glykosidartig vereinigt sind. Hiermit stimmen auch die Ergebnisse der Analysen überein.

0.0875 g lufttrockner Subst., im Vakuum über Phosphorsäureanhydrid auf 110° erhitzt, verloren 0.0100 g  $H_2O$ . — 0.1518 g lufttrockner Subst., im Vakuum über Phosphorsäureanhydrid auf 110° erhitzt, verloren 0.0167 g  $H_2O$ .

$C_{10}H_{13}O_5N_5 + 2H_2O$ . Ber.  $H_2O$  11.28. Gef.  $H_2O$  11.44, 11.00.

0.1351 g wasserfreie Subst.: 0.2078 g  $CO_2$ , 0.0557 g  $H_2O$ . — 0.0775 g wasserfreie Subst.: 17 ccm N (28°, 758 mm).

$C_{10}H_{13}O_5N_5$ . Ber. C 42.40, H 4.59, N 24.73.

Gef. > 41.94, > 4.58, > 24.91.

Die Substanz enthielt immer kleinere Mengen Asche, die nur sehr schwer entfernt werden konnten.

Im Capillarrohr rasch erhitzt, sintert sie bei 237° unter Verkohlen zusammen. In kaltem Wasser ist sie kaum löslich, aber leicht in heißem Wasser. In Mineralsäuren und in Alkalien löst sie sich ebenfalls leicht. Aus der alkalischen Lösung wird sie durch Essigsäure als Gallerte gefällt, in der sich allmählich schöne Krystallbüschel bilden. Für die optische Untersuchung wurde in Alkali gelöst.

0.1511 g der Sbst. in 47 ccm  $n_{10}$ -Natronlauge (1 Mol) gelöst. Gesamtgewicht der Lösung 4.8641 g. Spez. Gewicht 1.03. Drehte im 0.5-dm-Rohr bei Natriumlicht 0.94° nach links. Mithin

$$[\alpha]_D^{20} = -60.52^\circ.$$

Nach 48 Stunden war die Rotation dieselbe.

Aus der wäßrigen Lösung wird von Silbernitrat eine durchsichtige Gallerte gefällt, die in verdünnten Säuren und Alkalien leicht löslich ist. Von Bleiessig wird die wäßrige Lösung nicht gefällt, wohl aber nach Zusatz von Ammoniak. Aus den Mutterlaugen des Rohprodukts wurden durch fraktioniertes Füllen mit Bleiessig noch beträchtliche Mengen der Substanz erhalten.

Hydrolyse des Guanosins. 1.5 g Guanosin wurden in 150 ccm  $1/10$ -n. Schwefelsäure gelöst und eine Stunde am Rückflußkühler gekocht. Hiernach wurde das Guanin mittels Silbersulfat entfernt und das Filtrat durch Schwefelwasserstoff und Bariumcarbonat von Silber und Schwefelsäure befreit. Das Filtrat wurde dann unter vermindertem Druck zur Trockne verdampft und der Rückstand mit heißem, absolutem Alkohol ausgelaugt. Die alkoholische Lösung dunstete im Exsiccator über Schwefelsäure zu einem farblosen, süß schmeckenden Sirup ein, der nach Impfen mit einem Kryställchen Carnose nach einiger Zeit erstarrte und ganz fest wurde. Die Masse wurde mit wenig Alkohol verrührt, abgesaugt und mit Äther gewaschen. Der Zucker hatte ganz des Aussehen der Carnose. Im Capillarrohr erhitzt, schmolz er bei 85° (korr.), bei welcher Temperatur sich auch die Carnose verflüssigt.

0.1500 g Sbst. wurden in 4 ccm Wasser gelöst. Gesamtgewicht der Lösung 4.3641 g. Drehte im 0.5-dm-Rohr bei Natriumlicht 0.33° nach links. Mithin

$$[\alpha]_D^{20} = -19.2^\circ (\pm 0.4^\circ).$$

Für Carnose aus Carnin wurde  $-19.5^\circ$  gefunden.

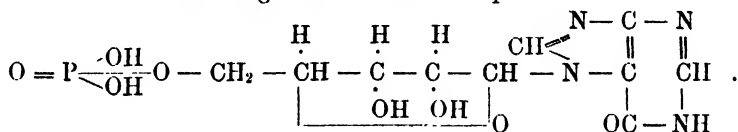


# 361. P. A. Levene und W. A. Jacobs: Über die Hefe-Nucleinsäure.

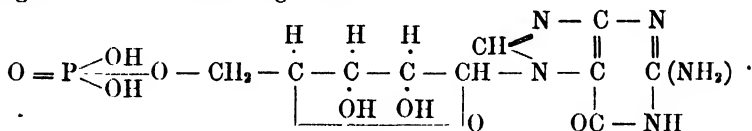
[Aus dem Rockefeller Institute for Medical Research, New York.]

(Eingegangen am 26. Mai 1909).

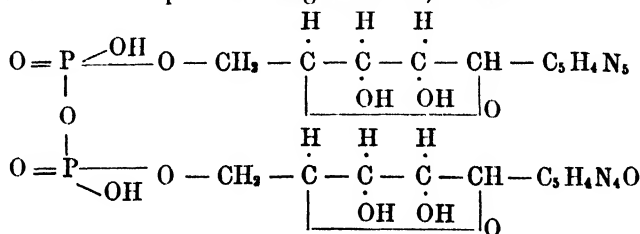
Wie bekannt, sind die komplizierteren Nucleinsäuren aus Purinbasen, Pyrimidinbasen, Kohlehydraten und Phosphorsäuren zusammengesetzt. Die einfacheren — Inosinsäure und Guanylsäure — bestehen nur aus je einer Purinbase, einer Kohlehydratgruppe und Phosphorsäure. Über die genauere Konstitution der Substanzen lagen bis vor kurzem keine experimentell sicher begründeten Ansichten vor. Die Auffassung von Schmiedeberg<sup>1)</sup>, nach welcher als Grundsubstanz der Nucleinsäure das Nucleotin anzusehen war, stützte sich hauptsächlich auf spekulative Gründe. Vor einem Jahre haben nun Levene und Mandel<sup>2)</sup> den Gedanken ausgesprochen, daß die komplizierteren Nucleinsäuren aus mehreren Fragmenten aufgebaut seien, welche die Zusammensetzung der einfachen Nucleinsäuren, der Inosinsäure und der Guanylsäure, besitzen. Über die Inosinsäure waren dann in diesem Laboratorium Untersuchungen im Gange; nach diesen dürfte ihre Konstitution etwa folgendem Schema entsprechen:



Aus denselben Gründen mußte die Guanylsäure dann die folgende Zusammensetzung haben:



Komplexe dieser Ordnung haben Levene und Mandel Mononucleotide genannt; die komplizierteren Nucleinsäuren sollten dann aus mehreren solchen Komponenten aufgebaut sein, etwa nach dem Schema:



<sup>1)</sup> Arch. f. Exp. Path. u. Pharmakol. **43**, 65 [1899]; Alsberg, *ibid.* **51**, 240 [1904].

<sup>2)</sup> Diese Berichte **41**, 1905 [1908].

Seitdem hat sich diese Ansicht über die Konstitution der Inosinsäure durch die Arbeiten von Levene und Jacobs<sup>1)</sup>, sowie von Haiser und Wenzel<sup>2)</sup> experimentell begründen lassen. Ganz vor kurzem<sup>3)</sup> ist es uns nun gelungen, auch für die Guanylsäure eine analoge Zusammensetzung zu beweisen. Unaufgeklärt war nur die Stellung derjenigen Hydroxylgruppe geblieben, durch welche Zucker und Phosphorsäure gebunden sind. Die Bindung der Purinbasen würde man wahrscheinlich mit Burian in der Stellung 7 annehmen müssen.

Vor einiger Zeit hat Levene<sup>4)</sup> Beweise für die Annahme beigebracht, daß auch die Hefe-Nucleinsäure eine Zusammensetzung nach demselben Schema wie die Thymonucleinsäure besitzt. Er stützte sich hierbei darauf, daß man bei partieller Hydrolyse mittels verdünnter Mineralsäuren zu Komplexen gelangt, die scheinbar aus Pyrimidinbasen, einer Pentose und Phosphorsäure zusammengesetzt wird, während man durch alkalische Hydrolyse glykosidartige Körper erhält. Zu jener Zeit war die Isolierung dieser Körper in rein krystallinischer Form noch nicht gelungen; jetzt aber, wo es ermöglicht worden ist, solche Körper zu gewinnen und genauer zu untersuchen, schlagen wir für sie die allgemeine Benennung »Nucleoside« vor. Zwei solcher Nucleoside sind schon jetzt bekannt geworden: Das Inosin, welches Haiser und Wenzel im Carnin entdeckten, und welches wir als Komponente der Inosinsäure erkannten, und das von uns entdeckte Guanosin<sup>5)</sup>, das eine Komponente der Guanylsäure darstellt.

Nun ist es uns jetzt gelungen, das Guanosin auch bei der Hydrolyse der Hefe-Nucleinsäure zu erhalten. Um diese Substanz in guter Ausbeute zu gewinnen, braucht man nämlich die Spaltung nur bei möglichst neutraler Reaktion der Lösung auszuführen. Dieser Befund enthält also den Beweis, daß die Hefe-Nucleinsäure aus mehreren Nucleotiden besteht, welche nach dem Typus der Guanylsäure zusammengesetzt sind.

Bei der Spaltung der Hefe-Nucleinsäure erhielten wir in der Mutterlauge vom Guanosin noch mehrere ähnliche Körper in amorpher Form. Diese Körper sind phosphorfrei und liefern bei der Hydrolyse mittels verdünnter Mineralsäuren die Pentose und Basen. Mit ihrer Bearbeitung sind wir jetzt beschäftigt.

Dieser Befund ermöglichte es uns auch, Auskunft über die Natur der in der Hefe-Nucleinsäure vorkommenden Pentose zu ge-

<sup>1)</sup> Diese Berichte **41**, 2703 [1908]; **42**, 335 [1909].

<sup>2)</sup> Monatsh. f. Chemie **29**, 157 [1908]; *ibid.* **30**, 147 (1909).

<sup>3)</sup> Vergl. die voranstehende Mitteilung.

<sup>4)</sup> Biochem. Zeitschr. **17**, 120 [1909].

<sup>5)</sup> Vergl. die voranstehende Mitteilung.

winnen. Auf Grund der Untersuchungen von Neuberg<sup>1)</sup> und seiner Mitarbeiter hat man die in der Nucleinsäure vorkommende Pentose als *l*-Xylose aufgefaßt. Es war zum ersten Male bei der Untersuchung der Inosinsäure, daß wir auf Widersprüche zwischen den Angaben von Neuberg und unseren Befunden über das Drehungsvermögen des Phenylsazons stießen; später ist es uns dann gelungen, die Unhaltbarkeit der Neubergschen Ansicht über die Natur der Pentose aus der Inosinsäure und der Guanylsäure definitiv zu beweisen. Es gelang uns nunmehr, diese Pentose in krystallinischer Form zu erhalten. Provisorisch hatten wir sie Carnose genannt, jetzt aber finden wir uns berechtigt, sie als *d*-Ribose anzusprechen. Ferner ist es uns nunmehr gelungen, dieselbe Pentose auch aus dem Guanotin der Hefe-Nucleinsäure zu erhalten. Auch bei der direkten Spaltung der Hefe-Nucleinsäure mittels verdünnter Mineralsäuren bildet sich die gleiche linksdrehende Pentose; ihre Isolierung ist aber hier mit vielen Mühen und Verlusten verbunden, so daß wir aus 30 g der Nucleinsäure nur etwa 0.35 g des reinen, in diesem Fall jedoch nicht krystallinischen Zuckers erhielten. Wie in früheren Mitteilungen erwähnt ist, begegnete man auch bei der Hydrolyse der Inosinsäure und der Guanylsäure ähnlichen Schwierigkeiten.

Wir sind jetzt mit der Darstellung der Nucleoside aus der Thymonucleinsäure beschäftigt, und hoffen, durch diese Untersuchung die Natur der dort vorkommenden Hexose aufzuklären.

### Experimenteller Teil.

**Darstellung der Hefe-Nucleinsäure.** Die für diese Arbeit gebrauchte Nucleinsäure wurde von Boehringer, Mannheim, bezogen. Die Substanz war nicht eiweißfrei; zu ihrer Reinigung wurde sie in möglichst wenig heißem Wasser gelöst und mit viel Eisessig gefällt. Die auf diese Weise erhaltene Substanz war ganz biuretfrei.

### Neutrale Hydrolyse der Hefe-Nucleinsäure.

20 g der reinen Hefe-Nucleinsäure wurden in 80 ccm 10-proz. Natronlauge unter Erwärmen aufgelöst. Die schwach gelb gefärbte Lösung wurde dann mit Essigsäure versetzt, bis sie auf Lackmuspapier amphotere Reaktion zeigte. Die Reaktion darf weder schwach sauer, noch alkalisch sein, da im ersten Falle beim Erhitzen vollständige Hydrolyse eintreten würde, gegen Alkali die Substanz aber sehr resistent ist. Die neutrale Lösung wird dann auf 500 ccm verdünnt und im Einschließrohr 6 Stunden auf 130—140° erhitzt. Nach dem Er-

<sup>1)</sup> Diese Berichte **32**, 3384 [1899].

kalten wurde die klare Lösung mit der dreifachen Menge Wasser verdünnt und im Wasserbade erhitzt. Die Lösung wurde dann mit Bleiessig versetzt, bis die Fällung gerade beendet war, der Niederschlag abgesaugt und mit Wasser nachgewaschen. Das Filtrat wurde mit Bleiessig und Ammoniak vollständig gefällt. Der Niederschlag wurde nach dem Abfiltrieren und sorgfältigem Auswaschen in 200 ccm Wasser aufgeschwämmt und mittels Schwefelwasserstoff vollständig zersetzt. Die Mischung wurde nach Zusatz von etwas Tierkohle zum Sieden erhitzt und rasch filtriert. Das Filtrat wurde unter vermindertem Druck auf 50 ccm eingeeengt, wobei es rasch zu einer gelatinösen Masse erstarrte, die im Eisschrank bald krystallisierte. Die Krystalle, die genau das Aussehen des Guanosins besaßen, wurden abfiltriert, sorgfältig gewaschen und aus möglichst wenig heißem Wasser umkrystallisiert. Das Produkt gab starke Pentosen-Reaktion, zeigte keinen gebundenen Phosphor und besaß nach der Hydrolyse reduzierende Eigenschaften. Im Capillarrohr rasch erhitzt, sinterte es bei 237° (korr.).

0.1508 g Subst. im Vakuum über  $P_2O_5$  getrocknet: 0.0169 g  $H_2O$ .

$C_{10}H_{13}O_5N_5 + 2 H_2O$ . Ber.  $H_2O$  11.28. Gef.  $H_2O$  11.21.

0.1339 g wasserfreie Subst.: 0.2061 g  $CO_2$ , 0.0597 g  $H_2O$ .

$C_{10}H_{13}O_5N_5$ . Ber. C 42.40, H 4.59.

Gef. » 41.97, » 4.95.

0.1277 g Subst. in 4 ccm (1 Mol.)  $\frac{1}{10}$ -n. NaOH gelöst; Gesamtgewicht der Lösung 4.2243 g. Drohte im 0.5-dm-Rohr mit Natriumlicht 0.90° nach links. Mithin

$$[\alpha]_D = -59.54^\circ (\pm 0.6^\circ).$$

Für Guanosin aus Guanylsäure war die Drehung  $[\alpha]_D = -60.52^\circ (\pm 0.6^\circ)$ .

Es ist also kein Zweifel mehr möglich, daß das Guanosin aus der Hefe-Nucleinsäure und das Guanosin aus der Guanylsäure identisch sind.

Durch die Hydrolyse ist dies dann noch weiter bewiesen worden. Die Mutterlauge vom Guanosin gab sehr starke Pentosen-Reaktion, zeigte aber keine reduzierenden Eigenschaften und war frei von gebundenem Phosphor. Sie enthielt aber ohne Zweifel noch andere Nucleoside, wie Adenosin und so weiter, die von Alkohol amorph gefällt wurden. Mit der Isolierung dieser Körper sind wir jetzt beschäftigt.

### Hydrolyse des Guanosins.

1.3 g Guanosin wurden genau nach der für das Guanylsäure-Guanosin angegebenen Methode hydrolysiert. In derselben Weise wurde der Zucker, die *d*-Ribose, in rein krystallinischer Form er-

halten. Zwecks Identifizierung wurde das Drehungsvermögen bestimmt.

0.1118 g Subst. in 3.5 ccm Wasser gelöst; Gesamtgewicht der Lösung 3.6833 g. Drehte im 0.5-dm-Rohr mit Natriumlicht  $-0.295^{\circ}$  nach links. Mithin  $[\alpha]_D = -19.44^{\circ}$ .

Der Purinbasen-Niederschlag, der mittels Silbersulfat erhalten worden war, wurde in verdünnter Schwefelsäure aufgeschwämmt und mittels Schwefelwasserstoff zersetzt. Nach dem Abfiltrieren schieden sich beim Erkalten des Filtrats schöne, lange Nadeln aus, die in allen Eigenschaften mit dem Guanin-Sulfat vollständig übereinstimmten.



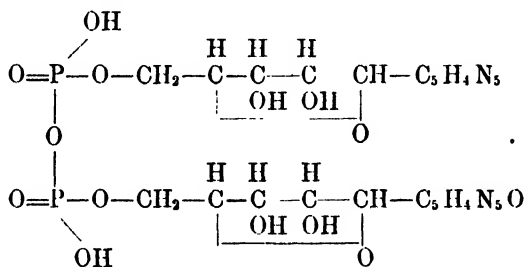
# 401. P. A. Levene und W. A. Jacobs: Über Hefenucleinsäure.

-(Zweite Mitteilung.)

[Aus dem Rockefeller Institute for medical Research, New York.]

(Eingegangen am 1. Juli 1909.)

Frühere Untersuchungen<sup>1)</sup> haben uns zur Annahme geführt, daß die Konstitution der Hefenucleinsäure die eines Polynucleotids ist. Als Mononucleotide bezeichnen wir Verbindungen aus je 1 Mol. Phosphorsäure, Pentose und Base. Diese Auffassung stützte sich auf die Befunde bei der Hydrolyse der Säure eines Komplexes, der aus Phosphorsäure, Pentose und Uracil zusammengesetzt war, und dann eines Komplexes, der aus Pentose und Guanin bestand, und den wir Guanosin genannt haben. Wir haben etwa das folgende Schema für die Zusammensetzung der Nucleinsäure formuliert:



Nach diesem Schema müssen 4 Nucleosid-(Kohlehydrat-Base)-Komplexe an dem Aufbau der Hefenucleinsäure teilnehmen.

In unserer früheren Mitteilung haben wir auch schon erwähnt, daß in der Mutterlauge vom Guanosin andere Nucleoside vorhanden waren. Jetzt ist es uns gelungen, den entsprechenden Adenin-Komplex — das Adenosin,  $\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_4$  — in schön krystallinischer Form zu erhalten. Die Substanz gleicht im Aussehen dem Guanosin, sie krystallisiert aber aus Wasser mit  $1\frac{1}{2}$  Molekülen Krystallwasser, besitzt den Schmp.  $229^\circ$  (korr.) und das Drehungsvermögen  $[\alpha]_D = -63.3^\circ$  in wäßriger Lösung.

In ihrer Löslichkeit in Wasser unterscheidet sich die Substanz vom Guanosin wesentlich, und auf dieser Eigenschaft beruht das Verfahren zur Trennung der beiden Substanzen.

Bei genauer Befolgung der im experimentellen Teil angegebenen Bedingungen läßt sich das Guanosin direkt aus dem Reaktionsgemisch fast quantitativ ausscheiden. Aus der Mutterlauge vom Guanosin

<sup>1)</sup> Diese Berichte 42, 2474 [1909].

läßt sich das Pikrat des Aldenosins in krystallinischer Form erhalten. Vom Pikrat aus kommt man dann zu dem freien Nucleosid.

Bei der Hydrolyse des Pikrats erhält man das Adeninpikrat in theoretischer Ausbeute, die Pentose erwies sich identisch mit der des Inosins oder des Guanosins, nämlich als *d*-Ribose<sup>1)</sup>.

### Experimenteller Teil.

#### Darstellung des Guanosins und Adenosins.

Wir lösten etwa 40.0 g frisch bereitete und über Nacht im Vakuum-exsiccator getrocknete Nucleinsäure in 120 ccm 10-proz. Natronlauge und neutralisierten die Lösung mit Essigsäure (Lackmus). Wir wollen nochmals auf die Wichtigkeit der neutralen Reaktion bei der Ausführung dieser Operation aufmerksam machen. Zu der neutralen Lösung wird dann 20.0 g Kaliumacetat zugefügt, die Lösung bis auf 1 l verdünnt und dann im Autoclaven 12 Stunden (Badtemperatur von 180–190°) erhitzt. Nach dem Abkühlen wurde das Reaktionsprodukt ein paar Stunden in einer Kältemischung stehen gelassen. Das Guanosin scheidet sich dabei fast quantitativ aus. Die Ausbeute an trockner Substanz betrug gewöhnlich 4.0 g.

Das Filtrat vom Guanosin wird mit einer 25-proz. Lösung von Bleizucker bis zur vollständigen Fällung behandelt, und aus dem Filtrat von dieser Fällung werden mittels Ammoniakwasser die Bleiverbindungen der Nucleoside gefällt. Diese wurden in Wasser suspendiert, mit Schwefelwasserstoff von Blei befreit und die Lösung zu einem dicken Sirup eingedampft. Letzteren trägt man wieder in eine Kältemischung ein, um Spuren von Guanosin auszuschcheiden. Bei genauer Beobachtung der oben angegebenen Bedingungen ist alles Guanosin schon durch die erste Fällung entfernt, und der Sirup setzt keinen Niederschlag ab, auch nach längerem Erkalten nicht. Zu diesem Sirup wird dann eine heiße gesättigte Lösung von Pikrinsäure (etwa 3.0 g) zugefügt, das Adenosinpikrat fällt nun als Gallerte aus. Nach einmaligem Umkrystallisieren erhielt man aber die Substanz in schönen, glänzenden Plättchen. Im Capillarrohr rasch erhitzt, fing sie bei 180° zu sintern an, und gegen 185° (korr.) ist sie völlig zusammengesunken. Die Ausführung der Verbrennung verlief ziemlich schwierig, und die analytischen Zahlen waren nicht ganz befriedigend.

0.1217 g Sbst.: 0.1695 g CO<sub>2</sub>, 0.0428 g H<sub>2</sub>O. — 0.1652 g Sbst.: 32 ccm N (21°, 753 mm).

C<sub>10</sub>H<sub>13</sub>N<sub>5</sub>O<sub>4</sub> · C<sub>6</sub>H<sub>3</sub>(NO<sub>2</sub>)<sub>3</sub>OH. Ber. C 38.71, H 3.25, N 22.58.

Gef. » 37.08, » 3.93, » 22.90.

Das Pikrat wurde dann in heißem Wasser gelöst, bald nach dem Abkühlen (nach längerem Stehen scheidet sich das Adenosinpikrat wieder aus) mit einem kleinen Überschuß von Schwefelsäure angesäuert und mit Äther

<sup>1)</sup> Die Gründe, aus denen wir die Pentose als *d*-Ribose — und nicht, wie Hauser und Wenzel (Monatsh. für Chem. 80, 377 [1909]) als *d*-Lyxose — auffassen, werden wir demnächst angeben.

ausgezogen. Die von Pikrinsäure befreite Lösung wird mit frisch vorbereitetem Bariumcarbonat erwärmt und das Filtrat von den Bariumverbindungen bis zu einem ganz kleinen Volumen bei vermindertem Druck eingedampft. Die Lösung wird dann im Vakuumexsiccator über Schwefelsäure weiter konzentriert, wobei bald die Krystallisation des freien Adenosins anfängt; nach einiger Zeit erstarrte die Lösung zu einem dicken Brei von langen, dünnen, tyrosinartigen Nadeln. Da die Analyse einen Aschengehalt von 2% zeigte, so wurde der Körper zweimal aus heißem Wasser unter Verwendung von Tierkohle umkrystallisiert. Der rein weiße Körper enthielt so nur Spuren von Asche und wurde an der Luft zum konstanten Gewicht getrocknet.

0.1563 g Subst. (im Vakuum über  $P_2O_5$  bei  $110^\circ$  erhitzt): 0.0143 g  $H_2O$ .

0.2324 » » » » » » »  $110^\circ$  » : 0.0214 » »

$C_{10}H_{13}O_4N_5 + 1\frac{1}{2}H_2O$ . Btr.  $H_2O$  9.18. Gef.  $H_2O$  9.15, 9.21.

0.1172 g wasserfreie Subst.: 0.1932 g  $CO_2$ , 0.0544 g  $H_2O$ .

$C_{10}H_{13}O_4N_5$ . Ber. C 44.94, H 4.87.

Gef. » 44.96, » 5.15.

Im Capillarrohre rasch erhitzt, schmilzt die Substanz bei  $229^\circ$  (korr.). In Wasser ist sie ziemlich löslich, besonders beim Erwärmen, und durch Verunreinigungen wird ihre Löslichkeit bedeutend erhöht. Von Alkohol wird sie kaum aufgenommen.

Zur optischen Bestimmung wurde zuerst eine wäßrige Lösung der krystallwasserhaltigen Substanz benutzt: aber da die Substanz nur beim Erwärmen gelöst werden konnte, verliert die Bestimmung etwas an Genauigkeit. Eine zweite Bestimmung wurde in alkalischer Lösung ausgeführt.

1. 0.2107 g Subst. in 4 ccm Wasser gelöst. Gesamtgewicht der Lösung 4.2225 g. Drehte mit Natriumlicht im 0.5-dm-Rohr  $1.58^\circ$  nach links. Mithin  $[\alpha]_D = -63.3^\circ$  ( $\pm 1.2$ ).

2. 0.1431 g wasserfreier Subst. in 5.5 ccm  $\frac{1}{10}$ -n. NaOH (1 Mol.) gelöst. Gesamtgewicht der Lösung 5.6648 g. Drehte mit Natriumlicht im 0.5-dm-Rohr  $0.85^\circ$  nach links. Mithin  $[\alpha]_D = -67.30^\circ$ .

Hydrolyse des Adenosins. Zuerst wurde das Pikrat hierfür benutzt. 1.5 g des Pikrats wurden in 800 ccm heißen Wassers unter Zugabe von 75 ccm  $\frac{n}{10}$ -Schwefelsäure aufgelöst und am Rückflußkühler 2 Stunden lang gekocht. Die Lösung blieb über Nacht stehen, wobei sich das Adeninpikrat ausschied. Nach einmaligem Umkrystallisieren zeigte die Substanz folgende Eigenschaften:

Im Capillarrohre erhitzt fängt sie gegen  $260^\circ$  an zu sintern, und bei  $296^\circ$  (korr.) zersetzt sie sich unter Gasentwicklung.

Zur Analyse wurde das lufttrockne Präparat benutzt.

0.1113 g Subst. im Vakuum über  $P_2O_5$  bei  $110^\circ$  erhitzt: 0.0050 g  $H_2O$ .

$C_5H_5N_5 \cdot C_6H_2(NO_2)_3.OH + H_2O$ . Ber.  $H_2O$  4.71. Gef.  $H_2O$  4.49.

0.1063 g wasserfreier Subst.: 28.2 ccm N ( $22^\circ$ , 767.5 mm).

$C_5H_5N_5 \cdot C_6H_2(NO_2)_3.OH$ . Ber. N 30.77. Gef. N 31.13.

Aus der Mutterlauge wurde, nach Entfernen der Pikrinsäure und des noch gelösten Adenins, unter Benutzung der üblichen Methode, der Zucker als Sirup erhalten; da er aber keine Neigung zum Krystallisieren zeigte, auch nicht nach dem Impfen, wurde das *p*-Bromphenylhydrazon dargestellt.

Der Körper war im Schmelzpunkt und Drehungsvermögen identisch mit dem Derivat der *d*-Ribose. Im Capillarrohre rasch erhitzt, sinterte er bei 166° und schmolz bei 170° (korr.).

0.1871 g Sbst. wurden in 5 ccm absolutem Alkohol gelöst. Gesamtgewicht der Lösung 4.1092 g. Drehte mit Natriumlicht im 0.5-dm-Rohr 0.12° nach rechts. Mithin  $[\alpha]_D = + 5.28^\circ (\pm 0.4)$ . Für das *d*-Ribose-Derivat ist  $[\alpha]_D = + 5.69^\circ (\pm 0.4)$

Aus dem Adenosin selbst wurde der Zucker rein erhalten. 0.9 g Adenosin wurden, wie bei Inosin und Guanosin angegeben, hydrolysiert. Der Zuckersirup krystallisierte nach dem Impfen leicht und diente zur optischen Bestimmung.

0.1556 g Sbst. in 4 ccm Wasser gelöst. Gesamtgewicht der Lösung 4.0472 g. Drehte mit Natriumlicht im 0.5-dm-Rohr 0.37° nach links. Mithin  $[\alpha]_D = - 19.25^\circ$ .

[Aus dem Kgl. preuß. Institut für Infektionskrankheiten in Berlin;  
Direktor: Geh. Obermedizinalrat Dr. Gaffky. Abteilungsvor-  
steher: Geh. Medizinalrat Prof. Dr. Wassermann.]

## **Ueber die Beziehungen von Enzymwirkungen zu den Erscheinungen der sogenannten Komplement- ablenkung bei Syphilis <sup>1)</sup>.**

Von **Wilfred H. Manwaring, M.D.**, (U.S.A.).

Mit 14 Figuren im Text.

(Eingegangen bei der Redaktion am 10. Juli 1909.)

### **I. Theoretischer Teil.**

Die von Bordet entdeckte Reaktion zwischen Bakterienemulsionen und dem Blut von mit den betreffenden Mikroorganismen infizierten Tieren bildet die Grundlage für die neueren Entdeckungen auf dem Gebiete der Immunitätschemie. Die Anwendung dieser Entdeckung auf die Syphilis und der Versuch, dieselbe auch auf andere Erkrankungen anzuwenden, hatten reichen Erfolg. Ich habe nun den Versuch unternommen, die verschiedenen auf Grund der ursprünglichen Bordetschen Entdeckung beobachteten Erscheinungen auf eine quantitative Grundlage zu bringen. Nur durch solches quantitatives Arbeiten erschien es möglich, die bei der Reaktion auftretenden einzelnen Erscheinungsfaktoren zu bewerten und die eigentliche Natur der Erscheinung zu bestimmen.

Diese Arbeiten wurden im Juli 1908 durch Krankheit unterbrochen und können jetzt nicht wieder aufgenommen werden. Deshalb halte ich es für angezeigt, die bisher erhaltenen Ergebnisse zu veröffentlichen; denn wenn die Resultate auch noch nicht genügen, um daraus bindende Schlüsse

1) Diese Arbeit wurde auf Grund einer "Fellowship" des Rockefeller Institute for Medical Research, New York City, ausgeführt.

zu ziehen, so gestatten sie doch, eine ganze Reihe von Vermutungen zu äußern, welche gerade in dem gegenwärtigen Stadium der Komplementbindungsforschungen von besonderem Werte sein können.

Als Material für die Untersuchungen wurden verwendet Hammelblutkörperchen, Meerschweinekomplement und Kaninchenambozeptor, wie diese für die laufenden Wassermann-Reaktionen im Institut gebraucht werden. Es erschien indessen notwendig, die gewöhnliche Untersuchungstechnik etwas abzuändern, um die Reaktion für quantitative Versuche brauchbar zu machen. Bei den gewöhnlichen Wassermann-Reaktionen wird die geringste zur vollständigen Hämolyse nötige Ambozeptorendosis durch Vorversuche festgestellt und der so gewonnene Titer bei den eigentlichen Versuchen in doppelter oder dreifacher Menge angewendet. Dadurch werden bei den eigentlichen Versuchen die roten Blutkörperchen einer Menge von Hämolsin ausgesetzt, die erheblich größer ist, als notwendig wäre, um eine vollständige Hämolyse herbeizuführen; infolgedessen sind geringere Schwankungen der lytischen Kräfte gar nicht festzustellen; es kommen vielmehr nur solche Veränderungen zur Beobachtung, welche zu einem vollständigen oder nahezu vollständigen Verlust dieser Fähigkeit führen. Eine Zunahme der lytischen Wirkungen, wie sie vielleicht bei einzelnen Versuchen erfolgen kann, ist so gar nicht festzustellen.

Fig. 1. Die Wassermannsche Reaktion. Die Kurve *ABb* stellt die Veränderungen dar, welche die hämolytische Wirksamkeit nach Zusatz wachsender Mengen verdünnten (10-proz.) luetischen Leberextraktes einem 88-proz. hämolytischen System (0,1 ccm Meerschweinchenkomplement + etwas weniger Ambozeptor, als dem ermittelten Titer entspricht) gegenüber unter den Bedingungen der gewöhnlichen Wassermannschen Reaktion erleidet. *ACc* gibt die entsprechenden Veränderungen bei Zusatz verdünnten (10-proz.), in der Hitze inaktivierten syphilitischen Menschenserums und *ADd* die bei normalem Serum wieder. Die steil abfallende Kurve zeigt bei jeder Figur die Erscheinungen, die bei gleichzeitiger Einwirkung von Extrakt und Serum auftreten. So sind bei *ABMN* (I) die geringen Veränderungen (*AB*) zu sehen, die anfangs bei Zusatz von Leberextrakt entstehen, bis eine plötzliche, fast vollständige Zerstörung der hämolytischen Wirkung erfolgt, sobald man nunmehr luetisches Serum zusetzt. *ABQR* (III) zeigt, daß eine Vernichtung der hämolytischen Wirksamkeit fehlt, wenn man ebenso normales Serum prüft. *ACOP* (II) und *ADST* (IV) zeigen einen ähnlichen Unterschied; hierbei ist die Methode insofern verändert, als der Leberextrakt bei gleichbleibender Serummenge zugesetzt wurde. Teil I und II der Figur zeigen eine positive, III und IV eine negative Wassermannsche Reaktion.

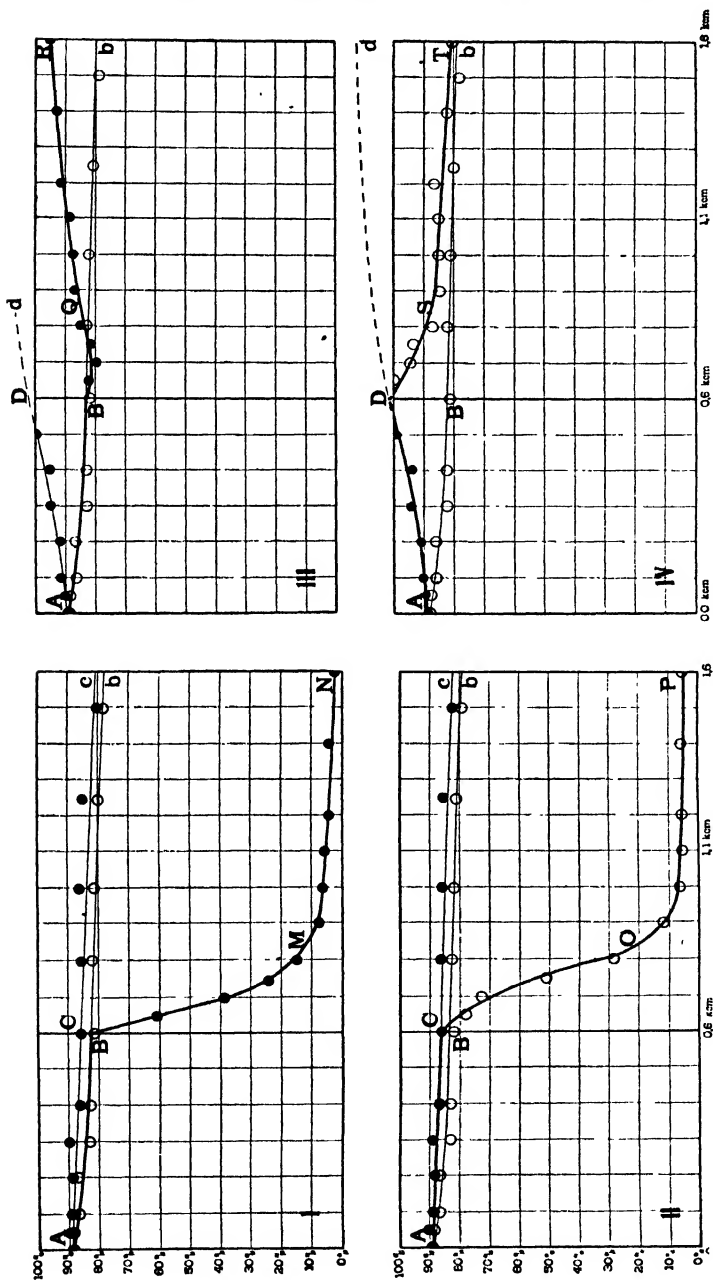


Fig. 1. Figurenerklärung siehe p. 310.

Diese ganze Erscheinung ließ sich in das Bereich einer quantitativen Bestimmung rücken, wenn man die bei den eigentlichen Versuchen zu nehmende Ambozeptordosis so weit reduzierte, daß sie dem festgestellten Titer gleich war, oder nur wenig größer als derselbe. Nach dieser Abänderung läßt sich die ganze Wassermannsche Reaktion graphisch darstellen. Eine solche Versinnbildlichung sieht man in der Fig. 1 (I, II); in derselben stellt die Kurve *ABb* die Veränderungen der hämolytischen Wirksamkeit<sup>1)</sup> dar, wenn man wachsende Mengen luetischen Leberextraktes dem Wassermannschen hämolytischen System zusetzt; *ACc* stellt in entsprechender Weise die Veränderungen bei Zusatz wachsender Mengen inaktivierten luetischen Serums dar, und *BMN* und *COP* das so gut wie völlige Ausbleiben der hämolytischen Wirkung (Wassermannsches Phänomen), wenn eine der beiden Substanzen bei Anwesenheit der anderen zugesetzt wird. Die Wirkung normalen Serums (negative Wassermannsche Reaktion) läßt sich ebenso leicht darstellen und findet sich in derselben Figur (III, IV). Hieraus ist bei den Kurven *BQR* und *DST* ersichtlich, daß die hämolytische Wirkung nicht wesentlich gehemmt ist, wenn normales Serum in Gegenwart von demselben Extrakt wirken kann.

Durch diese Abänderung der gewöhnlichen Wassermannschen Technik wird ein neuer Faktor in den Versuch nicht eingeführt; dies beweist Fig. 2; bei welcher die Er-

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1) Die hämolytische Wirksamkeit wird hierbei kolorimetrisch bestimmt und in Prozenten der vollständigen Hämolyse ausgedrückt. Wie bei den regelmäßigen Wassermannschen Reaktionen läßt man bei diesen Bestimmungen die zu untersuchende Substanz oder Substanzmischung eine Stunde lang im Brutschrank auf das Komplement einwirken; dann erst werden die sensibilisierten Blutkörperchen zugesetzt. Diese Gemische läßt man 2 Stunden lang bei 37,5° stehen. Nun stellt man sie in einen Kühlschrank, bis die ungelösten Blutkörperchen zu Boden gesunken sind (gewöhnlich 18 Stunden). Darauf wird die kolorimetrische Bestimmung vorgenommen. Die bei diesen Versuchen verwendeten Mengen sind mit geringen Abweichungen dieselben wie bei den laufenden Wassermannschen Reaktionen, nämlich: 1 ccm 10-proz. Meerschweinchenkomplement, 1 ccm Leberextrakt, 1 ccm 10-proz. inaktiviertes Menschenserum und 2 ccm 5-proz. sensibilisierte Hammelblutkörperchen (= 1 ccm 10-proz. Hammelblutkörperchen + 1 ccm verdünnte Ambozeptoren).

scheinungen parallel aufgeführt sind, je nachdem die Ambozeptorendosis zwischen dem Vierfachen des bestimmten Titors ( $A_4C_4O_4P_4$ ) und seiner Hälfte ( $A_0C_0O_0P_0$ ) schwankt. Diese vier hier dargestellten Kurven haben so ziemlich das gleiche

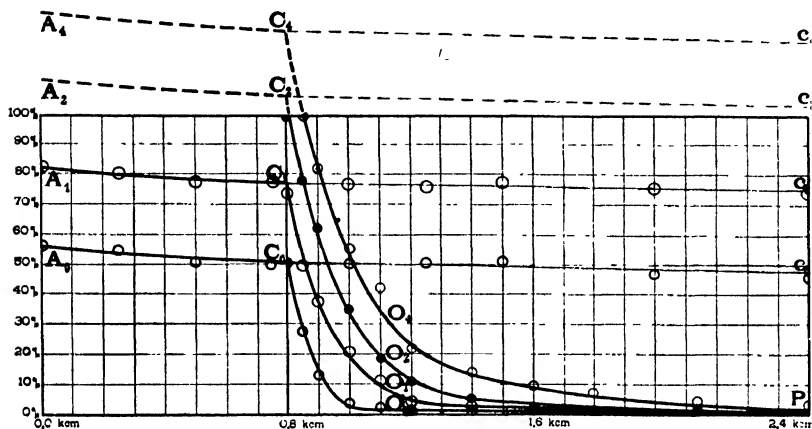


Fig. 2. Einfluß der Ambozeptormenge.  $A_4C_4O_4P$ ,  $A_2C_2O_2P$ ,  $A_1C_1O_1P$  und  $A_0C_0O_0P$  zeigen die hämolysenzerstörende Wirkung des Leberextraktes, der bei Anwesenheit gleichbleibender Mengen von luetischem Serum<sup>1)</sup> einwirkt, während die zugesetzten Blutkörperchen mit der vierfachen, doppelten, einfachen und halben Menge des ermittelten Ambozeptorentiters sensibilisiert sind. Die Kurve  $A_2C_2O_2P$  zeigt den Vorgang bei der gewöhnlichen Wassermannschen Methode,  $A_1C_1O_1P$  den bei der Methode, wie sie zu den vorliegenden Untersuchungen verwendet wurde. Beide Kurven zeigen im wesentlichen dieselben Erscheinungen und rechtfertigen damit die angewendete Methodik.

Aussehen; der einzige Unterschied zwischen ihnen ist der, daß bei den größeren Ambozeptorendosen, wo im Anfang die hämolytische Wirksamkeit 100 Proz. erheblich übersteigt<sup>2)</sup>,

1) Soweit nichts anderes bemerkt ist, sind bei diesen Versuchen alle Seren und Extrakte in 10-proz. Verdünnung verwendet worden.

2) Wenn die vorhandene Menge des Hämolysins größer ist, als für eine vollständige Lyse erforderlich ist, kann man ungefähr in ziemlich roher Weise die hämolytische Wirksamkeit abschätzen, wenn man die Reaktionszeit in Betracht zieht. Bei der hämolytischen Minimaldosis stellt sich die vollständige Hämolysen in  $1\frac{1}{2}$  bis 2 Stunden ein, während bei größeren Dosen die Lyse binnen viel kürzerer Zeit vollständig ist. Wenn man alle Viertelstunden nachsieht, kann man sich ungefähr ein Bild davon machen, wie die durch kolorimetrische Methoden nicht unmittelbar bestimmbaren Teile der Kurve aussehen.

ein nicht unbeträchtlicher Teil der Kurven hat theoretisch ergänzt werden müssen (punktierte Linie), während man bei den kleineren Ambozeptorendosen die Kurven direkt verfolgen kann.

Wenn man versucht, für die Wassermannsche Reaktion einen sicheren Maßstab zu finden, so liegt die größte zu

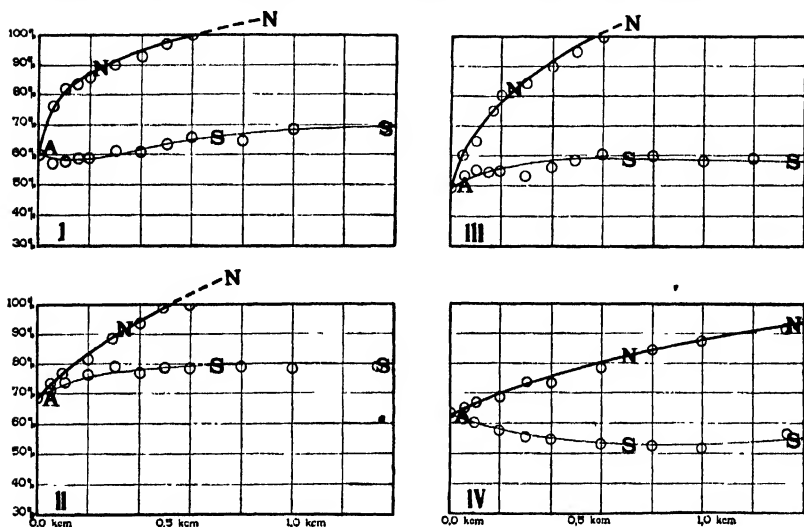


Fig. 3. Normale Serumauxilysine. Diese vier Versuche zeigen, daß normales, in der Hitze inaktiviertes Menschenserum eine verhältnismäßig starke hämolysenfördernde Wirkung hat (ANN), während bei den Kontrollversuchen mit inaktiviertem luetischen Serum diese Wirkung fehlt (ASS). Das Fehlen normaler auxilytischer Körper bei luetischen Seren spielt vermutlich bei der Wassermannschen Reaktion eine hervorragende Rolle.

überwindende Schwierigkeit in der mangelnden Konstanz und Haltbarkeit der Leberextrakte. Durch Anwendung der oben beschriebenen Methodik ließen sich Fingerzeige gewinnen, wie man Mittel zur Ausschaltung der Schwierigkeit finden könnte. Es wurde beobachtet, daß Seren, welche eine negative Wassermannsche Reaktion geben<sup>1)</sup>, unabhängige auxilytische oder hämolysenfördernde Kräfte besitzen (ANN

1) Für Ueberlassung der zu diesem Teil der Arbeit gebrauchten Seren, sowie die Diagnose bezüglich ihrer Reaktion bei der Wassermannschen Methodik bin ich dem Institutsassistenten, Herrn Dr. Georg Meier, zu Dank verpflichtet.

Fig. 3), während Wassermann-positive Seren solche Kräfte nicht aufweisen.

Indessen genügt die Zahl der auf die beschriebene Weise geprüften Seren noch nicht, um daraus Schlüsse auf die Gleichmäßigkeit oder Ungleichmäßigkeit des beobachteten Parallelismus ziehen zu können. Jedoch spricht für die Wahrscheinlichkeit, daß dieser Parallelismus gleichmäßig auftritt, und ferner dafür, daß die Wassermannsche Reaktion

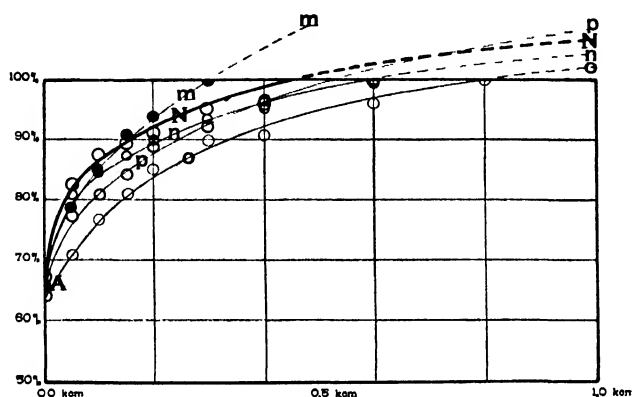


Fig. 4. Auxilytische Wirkung bei Anwesenheit von Leberextrakt. Durch Vorversuche wurden fünf hämolytische Systeme ausgesucht, die annähernd gleiche hämolytische Wirksamkeit (65-proz.) bei verschiedenem Gehalt anluetischem Leberextrakt zeigten. *ANN* zeigt die hämolysefördernde Wirkung von inaktiviertem normalen Menschenserum, das zu einem hämolytischen System zugesetzt ist, welches keinen Leberextrakt enthält. *Amm*, *Ann*, *Aoo* und *App* weisen annähernd gleiche Erscheinungen auf, bei einem Gehalt des hämolytischen Serums von 0,15, 0,3, 0,6 und 1,0 cm Leberextrakt. Die Tatsache, daß das Auxilysin im normalen Serum in Gegenwart von Leberextrakt aktiv ist, beweist, daß der normale auxilytische Körper bei der Wassermannschen Reaktion ein nicht zu vernachlässigender Faktor ist.

wenigstens teilweise von einem Mangel derluetischen Seren an normalen auxilytischen Körpern abhängig ist, die Beobachtung, daß dieses Auxilysin in Gegenwart vonluetischem Leberextrakt seine Wirksamkeit entfaltet (Fig. 4). Sollte dieser Parallelismus und diese kausalen Beziehungen endgültig bestätigt werden, so würde dies gestatten, die jetzt übliche Wassermannsche Methodik zu vereinfachen, indem der inkonstante, wenig haltbare Leberextrakt gänzlich entbehrlich würde, und an die Stelle der verhältnismäßig recht kompli-

zierten Komplementbindungsreaktion die relativ einfache Bestimmung der auxilytischen Wirkung träte<sup>1) 2)</sup>.

Des weiteren konnte beobachtet werden, daß zwischen der auxilytischen Wirkung normalen Serums und der Wirkung gewisser schwacher Alkalien eine Aehnlichkeit besteht, die vielleicht einen Parallelismus zwischen dem Alkalitäts- oder Aziditätsgrade erhitzter Seren<sup>3)</sup> und ihrem Verhalten bei der Wassermannschen Reaktion vermuten lassen könnte. Sollte sich auch dieser Parallelismus bestätigen, so könnte man sogar an die Möglichkeit denken, die hämolytische Reaktion ganz wegzulassen und dafür eine einfache chemische Titration vorzunehmen.

Ferner ließ sich feststellen, daß der Wassermannschen Reaktion quantitativ ganz gleiche Vorgänge sich auch mit unerhitztem Pferde-, Rinder- und Ziegenserum, sowie mit zahlreichen Laboratoriumssubstanzen erzielen lassen. Die Körper, welche solche Reaktionen geben, gehören hauptsächlich in zwei Gruppen: 1) Säuren und 2) Körper, welche Kofermente oder Fermentstimulatoren enthalten<sup>4)</sup>.

1) Falls jemand diesen Parallelismus näher nachprüfen will, so ist es rätlich, zu auxilytischen Bestimmungen eine Ambozeptorendosis zu wählen, welche für eine 30-proz. Hämolyse genügt. Es konnte beobachtet werden, daß ein 30-proz. hämolytisches System eine maximale Empfindlichkeit gegenüber Auxilysinen hat.

2) Anmerkung während der Korrektur. Diese auxilytische Substanz ist vermutlich identisch mit den Hammelblutkörperchen-Ambozeptoren, die kürzlich von verschiedenen Autoren beschrieben worden sind. Später vorgenommene Bestimmungen haben den Beweis erbracht, daß die Menge dieses Ambozeptors, welche bei verschiedenen Proben menschlicher Seren erheblichen Schwankungen unterliegt, zu der Reaktion des Serums bei der Wassermannschen Probe in keiner konstanten Beziehung steht.

3) Bei 55—56° eine halbe Stunde lang inaktiviert.

4) Die Wirksamkeit körperlicher Enzyme hängt wahrscheinlich stets ab von der Anwesenheit mindestens zweier chemischer Körper: 1) einem an sich inaktiven thermolabilen Ferment und 2) einem beständigeren Koferment oder Fermentaktivator. Die Natur dieser Kofermente ist noch unbekannt, jedoch vermutet man, daß einige davon mit dem Lecithin nahe verwandt sind (Glyzerophosphate). Unter einem Fermentstimulator versteht man einen Körper, der zwar an sich zu einer Enzymwirkung nicht unbedingt nötig ist, diese Wirkung aber erheblich befördern kann. Als Beispiel für eine Fermentstimulation sei die Wirkung des Natriumphosphats auf die proteolytischen Enzyme in Hefepreßsaft angeführt.

Diese Beobachtung genügt, um die Aufstellung des Satzes zu gestatten, daß die wirksamen Prinzipien im Serum und Leberextrakt die in ihnen enthaltenen Säuren, Kofermente und Fermentstimulatoren sind, insbesondere die Aminosäuren und einfachen Polypeptide, die bei der Autolyse der Leber entstehen, sowie die Konfermente der Zelltätigkeit. Sollte dieser Satz bestätigt werden, so würde man daraufhin nicht nur eventuell den unzuverlässigen Leberextrakt durch eine Anzahl gleichmäßigerer Mischungen von organischen Säuren und synthetischen Kofermenten ersetzen können, sondern auch neue Gesichtspunkte bezüglich der Art der Vorgänge bei der sogenannten Ablenkung und ein anderes Bild von seiner angenommenen Spezifität oder Nichtspezifität bei Krankheiten gewinnen.

Durch Zufall wurde beobachtet, daß die meisten gewöhnlichen Bakteriennährböden zu den Substanzen gehören, die eine solche Reaktion geben können: dies verdanken sie wahrscheinlich ihrem hohen Peptongehalt. Diese Beobachtung legt die Vermutung nahe, daß beim Arbeiten mit Bakterienemulsionen und mit den bakteriellen Stoffwechselprodukten Versuchsfehler leicht unterlaufen können.

Quantitative Versuche über Autolyse von Meerschweinchenkomplement weisen darauf hin, daß in Meerschweinchen Serum wahrscheinlich ein proteolytisches Ferment vorhanden ist, welches innerhalb der Zeit und bei den Verhältnissen in der Wassermannschen Reaktion das Komplement vollständig zu zerstören imstande ist. Diese Autolyse ist so deutlich, daß man sich zu der weiteren Vermutung veranlaßt sehen kann, die Abnahme der hämolytischen Wirksamkeit bei der Wassermannschen Reaktion sei nicht auf eine Bindung des Komplementes durch Antikörper, sondern auf eine Zerstörung des Komplementes durch autolytische Enzyme zurückzuführen, welche von Säuren, Kofermenten und Fermentstimulatoren des menschlichen Serums und des Leberextraktes aktiviert und unterstützt wurden.

Diese Vermutung findet eine Stütze in der Beobachtung, daß die Anwesenheit des Ambozeptors während des Vorganges der Bindung das Komplement nicht schützt (Fig. 5), wie er es wohl tun müßte, wenn der Vorgang lediglich in einer

Bindung von Komplement an andere Körper bestünde. In-  
dessen verschwinden sowohl Komplement als Ambozeptor als  
solche aus der Lösung. Die Beobachtung rollt gleichfalls die  
Frage auf, ob die Wassermannsche Reaktion tatsächlich,  
wie allgemein angenommen wird, lediglich in einer Einwirkung

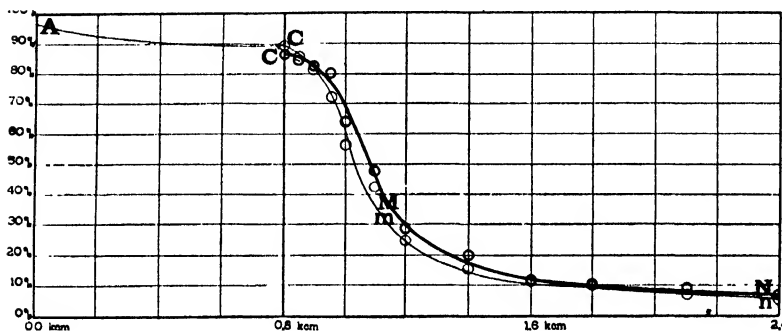


Fig. 5. Einfluß des Ambozeptors auf den Vorgang der Ablenkung. *ACmn* stellt die gewöhnliche Komplementablenkungsreaktion mit Meerschweinchenkomplement, Leberextrakt und luetischem Serum dar, während *C'MN* den gleichen Vorgang mit Meerschweinchenkomplement und der doppelten Ambozeptorentiterdosis veranschaulicht. Die Tatsache, daß die beiden Kurven innerhalb der Grenzen von Versuchsfehlern parallel verlaufen, beweist 1) daß die Gegenwart eines Ambozeptors das Komplement nicht vor dem Vorgang der Ablenkung schützt, und 2) daß der Ambozeptor seinerseits auch abgelenkt oder zerstört werden kann.

auf das Komplement besteht oder ob sie nicht eine kombinierte Einwirkung auf Komplement und Ambozeptor einschließt. Diese doppelte Wirksamkeit ist wohl möglich, da ja die sensibilisierten Blutkörperchensuspensionen viele freie Ambozeptoren enthalten.

Weiterhin wurde gefunden, daß es eine Pseudoablenkung gibt, eine Häufungserscheinung, die leicht für den Vorgang selbst gehalten werden kann. Diese Pseudoerscheinung kann eventuell zu Versuchsfehlern und Trugschlüssen Veranlassung geben. Auch ließ sich beobachten, daß wahrscheinlich ganz bestimmte mathematische Beziehungen zwischen den reagierenden Körpern bestehen, die auf die Annahme physikalisch-chemischer Gesetze hinweisen.

Am Ende dieser Uebersicht sei jedoch noch einmal betont, daß die aufgeführten Gesichtspunkte lediglich als vorläufige Hypothesen für fernere Arbeiten, nicht aber als be-

wiesene Tatsachen gelten sollen. Ihre Veröffentlichung in einem solchen unfertigen Zustande wurde nur in dem Sinne vorgenommen, daß sie vielleicht als Richtlinien für weitere Arbeiten für die künftige Entwicklung der Komplementbindungstheorie nicht ohne Wert sein mögen.

## II. Experimenteller Teil.

### 1. Versuche mit menschlichem Serum.

Diese Versuche mit menschlichem Serum wurden vor allem angestellt, um eine für quantitative Bestimmung brauchbare Technik zu gewinnen. Ueber diese Versuche enthält der theoretische Teil dieser Mitteilung die hauptsächlichsten Angaben. Der Unterschied zwischen normalem undluetischem Serum bei Anwendung der eingeschlagenen Methodik ist in Fig. 1 dargestellt. Fig. 2 enthält einen Vergleich der bei dieser Methode gewonnenen Resultate mit den Ergebnissen der gewöhnlichen Technik; daraus erhellt, wie berechtigt die eingeschlagene Methodik ist. Aus Fig. 3 geht hervor, daß erhitztes normales Serum einen verhältnismäßig starken Körper enthält, und daß dieser Körper beiluetischen Seren gänzlich oder nahezu gänzlich fehlt, oder bei ihnen sogar durch einen schwachen antilytischen Körper ersetzt ist. Fig. 4 zeigt, daß dieses normale Auxilylin bei Anwesenheit vonluetischem Leberextrakt wirksam ist, und daß es demnach mehr oder weniger für den Unterschied verantwortlich zu machen ist, den man bei der Wassermannschen Methode zwischen der Wirkung normalen undluetischen Serums beobachten kann. Aus Fig. 5 endlich erhellt, daß die Anwesenheit eines Ambozeptors während des Vorganges der Ablenkung das Komplement nicht nur nicht schützt, sondern daß der Ambozeptor sowohl als auch das Komplement während dieses Vorganges gebunden oder zerstört werden.

Diese Versuche führten zu zwei grundlegenden Hypothesen: erstens, daß der positive Ausfall einer Wassermannschen Reaktion wenigstens zum Teil auf dem Fehlen normaler auxilytischer Körper in dem betreffenden Serum beruht, und zweitens, daß die Reaktion nicht eine Wirkung des Komplementes

allein, sondern eine kombinierte Wirkung von Komplement und Ambozeptor ist.

Ueber die Natur dieser normalen Auxilysine ist eine Hypothese nicht aufgestellt; jedoch die Aehnlichkeit zwischen ihrer Wirkungsweise und der gewisser Alkalien<sup>1)</sup> weist darauf hin, daß man sie einmal wird als alkalische Körper nachweisen können, die entweder im menschlichen Serum normalerweise vorhanden sind oder in ihm bei dem Vorgang der Inaktivierung in der Hitze gebildet werden. Eine solche angenommene Differenz der Alkalität könnte weiterhin verantwortlich gemacht werden für den Unterschied, den man zwischen der Wirkung normalen undluetischen Serums bei Anwesenheit von schwachen Säuren beobachten kann, wobei das stärker alkalische Serum die Säuren neutralisieren und Säure wie Serum inaktiv werden könnten.

## 2. Ablenkungserscheinungen bei tierischen Seren.

Es konnte beobachtet werden, daß eine Anzahl tierischer Seren Ablenkungserscheinungen boten, die quantitativ der Wassermannschen Reaktion gleich waren. Da man solche Seren leicht in großen Mengen erhalten kann, liefern sie ein für quantitative Versuche ganz besonders geeignetes Material. Es ließ sich erwarten, daß die bei solchen Bestimmungen gewonnenen Ergebnisse zur Aufklärung der Natur der Erscheinungen bei der Wassermannschen Reaktion beitragen würden.

Die Figuren 6, 7 und 8 geben die Resultate einer Anzahl solcher Bestimmungen mit Pferde-, Rinder- und Ziegen-serum wieder. Aus diesen Versuchen geht hervor, daß man eine der Syphilisreaktion entsprechende Ablenkungserscheinung

Fig. 6. Ablenkungserscheinungen bei Pferdeserum. *ACc* (I) zeigt die Wirkung von 5-proz. Pferdeserum für sich allein, *ACEE* die Ablenkungserscheinung von 100-proz. menschlichem *Speichel*, wenn er in Gegenwart gleichbleibender Mengen von diesem Serum einwirkt, und in *Aee* ist die Wirkung dieses Speichels für sich allein dargestellt. Die übrigen untersuchten Stoffe sind unten zusammengestellt, wobei diejenigen, bei denen eine Ablenkungserscheinung auftritt, durch *Kursivschrift* hervorgehoben sind.

1) Vergl. den folgenden Abschnitt.

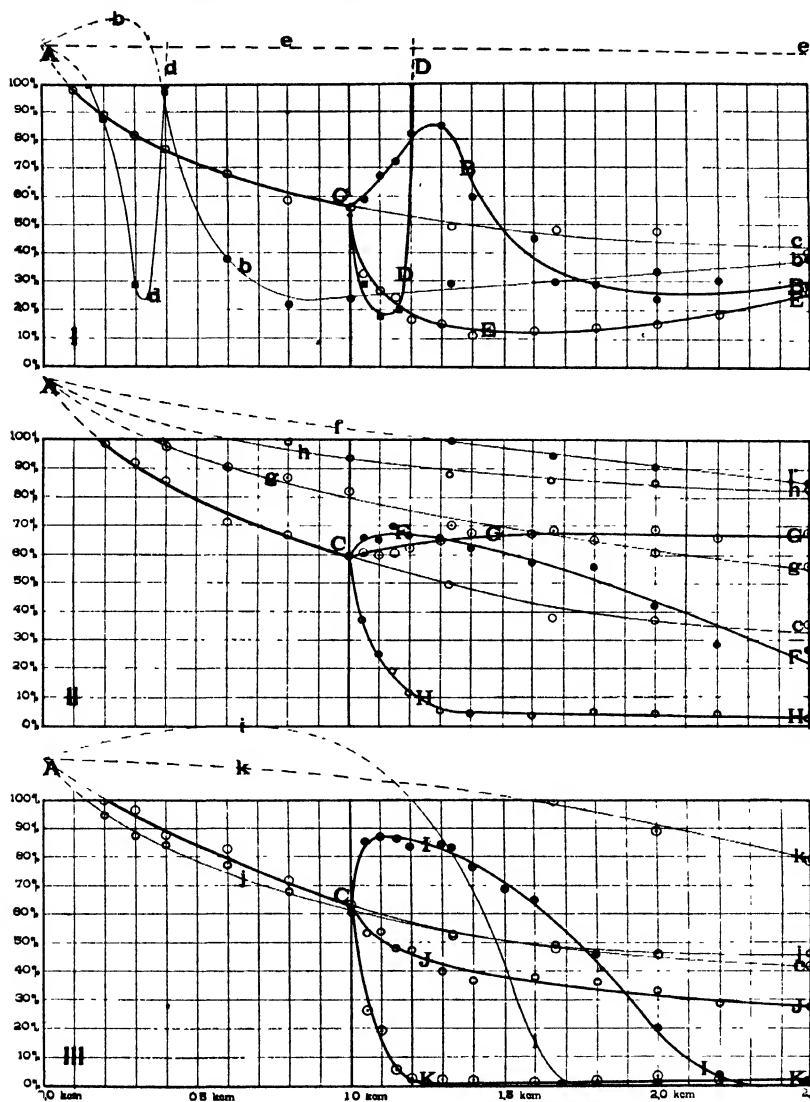


Fig. 6. Teil I—III. Figurenerklärung siehe p. 320.

I. Abb, ACBB =  $\frac{n}{40}$   $\text{Na}_2\text{CO}_3$ ,

II. Ajj, ACJJ = 1-proz.  $\text{MgCl}_2$ ,  
Agg, ACGG = 10-proz. Rinder-  
serum,

Add, ACDD =  $\frac{n}{40}$  Salzsäure.

Ahh, ACHH = 5-proz. Tuber-  
kulin.

III. Aii, ACII =  $\frac{1}{2}$ -proz. Trypsin,  
Ajj, ACJJ = 5-proz. Ziegen-  
serum,  
Akk, ACKK =  $\frac{1}{60}$ -proz. Lecithin.

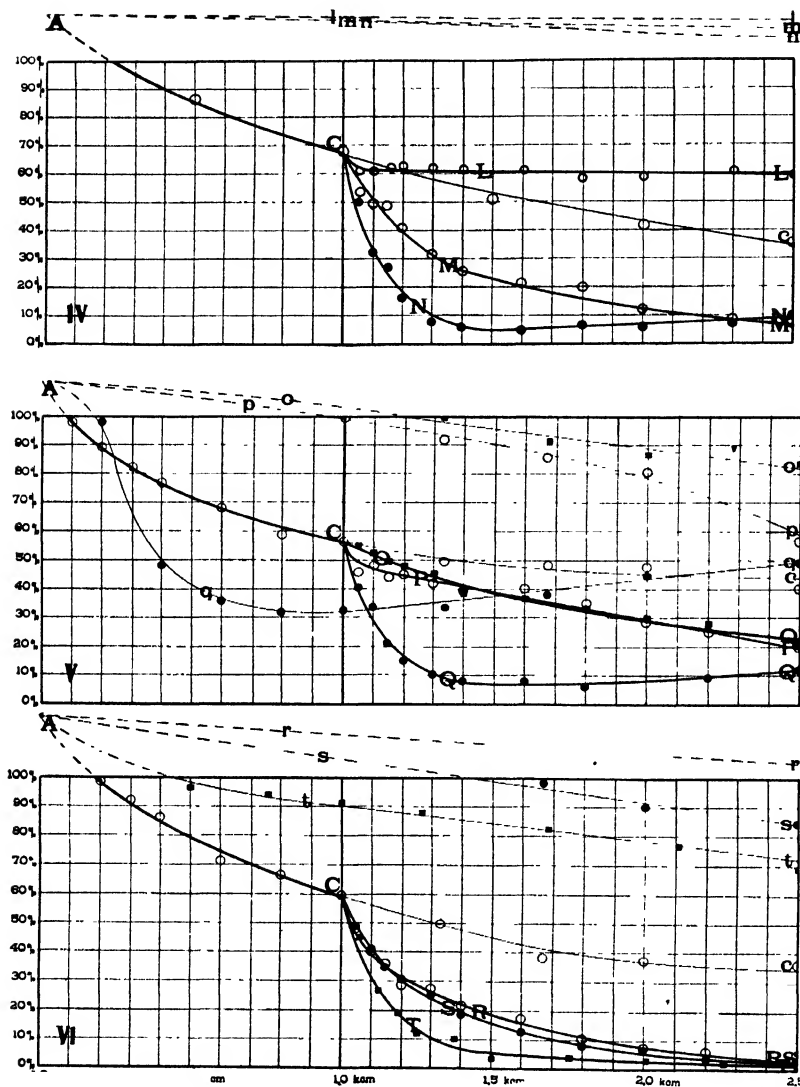


Fig. 6. Teil IV—VI. Figurenerklärung siehe p. 320.

IV. *Au*, *ACLL* = 1-proz. Glycerin,  
*Amm*, *ACMM* =  $\frac{1}{2}$ -proz. Gly-  
 kokoll,  
*Ann*, *ACNN* = 10-proz. Malz-  
 extrakt.

V. *Aoo*, *ACOO* =  $\frac{1}{8}$ -proz.  $\text{CaCl}_2$ ,  
*App*, *ACPP* =  $\frac{1}{35}$ -proz. grüne  
 Seife (unrein),  
*Aqq*, *ACQQ* = 10-proz. lueti-  
 scher Lebereextrakt.

VI. *Arr*, *ACRR* = 10-proz. Milch,  
*Ass*, *ACSS* =  $\frac{1}{3}$ -proz. Pepsin,  
*Au*, *ACTT* =  $\frac{1}{8}$ -proz. Labferment.

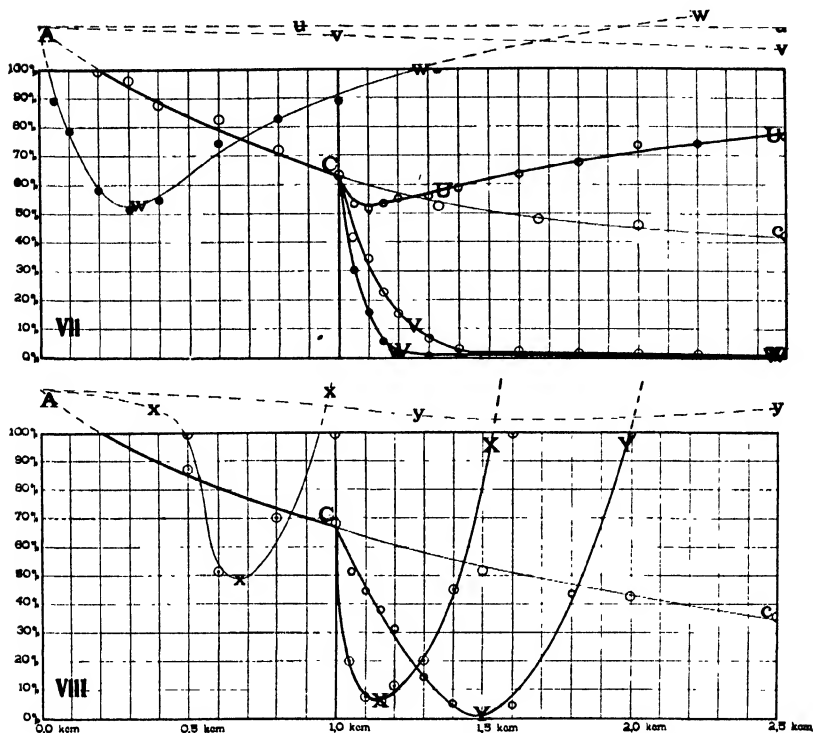


Fig. 6. Teil VII—VIII. Figurenerklärung siehe p. 320.

VII. *Auu*, *ACUU* = 10-proz. Ei-  
albumin,  
*Avv*, *ACVV* = 40-proz. Bouil-  
lon,  
*Aww*, *ACWW* = 5-proz. Hefe-  
preßsaft.

VIII. *Axx*, *ACXX* =  $\frac{n}{50}$  Phosphor-  
säure,  
*Ayy*, *ACYY* =  $\frac{1}{10}$ -proz. Oel-  
säure.

erhalten kann mit diesen Seren<sup>1)</sup> und Bouillon, Glykokoll, Hefepreßsaft<sup>2)</sup>, Lecithin, Leberextrakt, Malzextrakt, Milch, Pepsin (erhitzt und unerhitzt), Pepton, Lab (erhitzt und un-

1) Diese Seren wurden nicht durch Hitze inaktiviert, wie bei der gewöhnlichen Wassermannschen Technik, da sich herausstellte, daß keines von ihnen mit seinem Komplement Kaninchenambozeptoren aktivieren kann. Diese Aenderung der Technik hat, wie in einem späteren Abschnitt gezeigt werden soll, keine wesentliche Aenderung der Vorgänge zur Folge.

2) Für Ueberlassung des zu diesen Versuchen verwendeten Hefepreßsaftes bin ich Herrn Professor Eduard Buchner (Kgl. Landwirtschaftliche Hochschule in Berlin) zu Dank verpflichtet.

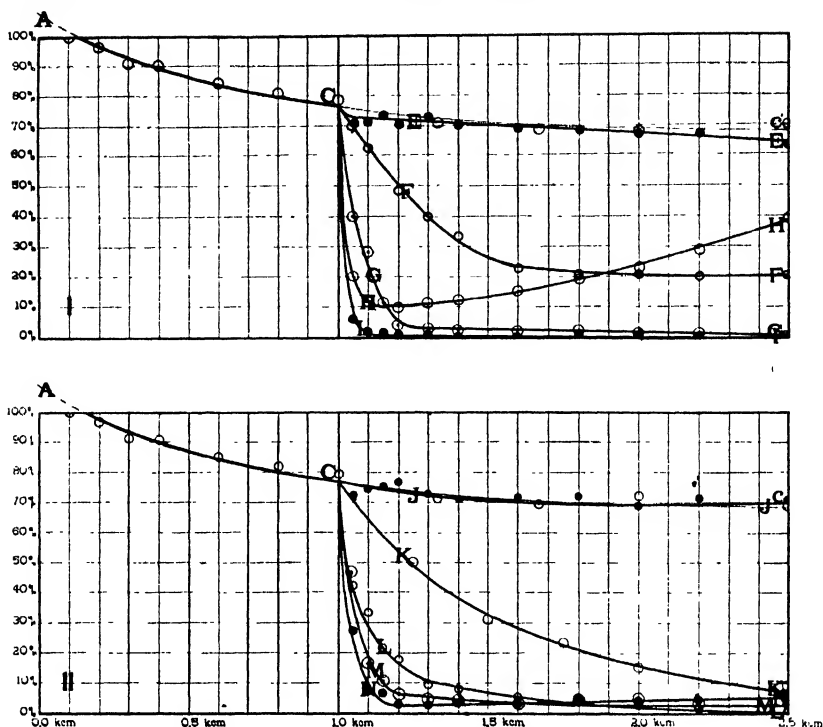


Fig. 7A. Ablenkungserscheinungen bei Rinderserum. Positive Ablenkungserscheinungen wurden in Gegenwart gleichbleibender Mengen von (10-proz.) Rinderserum beobachtet bei folgenden, durch *Kursivschrift* gekennzeichneten Stoffen:

I. *ACEE* = 1-proz. Stärke,  
*ACFF* = 10-proz. Milch,  
*ACGG* =  $\frac{1}{10}$ - „ Labferment,  
*ACHH* = 75- „ Speichel,  
*ACII* = 1- „ Pepton.

II. *ACJJ* =  $\frac{1}{2}$ -proz. Gelatine,  
*ACKK* =  $\frac{1}{10}$ - „ Agar,  
*ACLL* =  $\frac{1}{2}$ - „ Pepsin,  
*ACMM* = 5- „ Tuberkulin,  
*ACNN* = 50- „ Bouillon.

erhitzt), Speichel, Tuberkulin und in geringerem Grade reinem Agar und erhitztem Hefepreßsaft.

Einen der Syphilisreaktion ziemlich analogen, jedoch etwas komplizierteren Vorgang konnte man erzielen mit Oelsäure, Phosphorsäure, und etwas schwächer mit Salzsäure. Keine Reaktion trat ein mit: Eialbumin, Calciumchlorid, reiner Gelatine, Glyzerin, ölsaurem Kalium (unrein) und Stärke und mit Serum einer Tierart, wenn es in Gegenwart von Serum einer anderen Art wirkte. Eine der Syphilisreaktion entgegen-





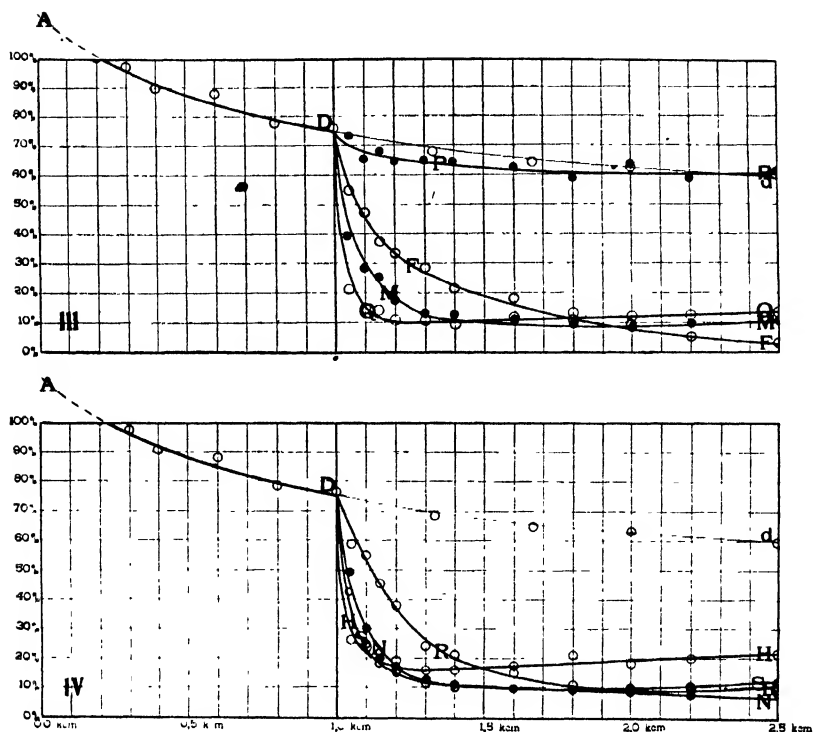


Fig. 7B. Ablenkungserscheinungen bei Ziegenserum. Positive Ablenkungserscheinungen wurden in Gegenwart gleichbleibender Mengen von (6-proz.) Ziegenserum beobachtet bei folgenden, durch *Kursiv-schrift* gekennzeichneten Stoffen:

III. *ADPP* = 10-proz. Rinder-serum,  
*ADFF* = 10-proz. Milch,  
*ADMM* = 5-proz. Tuberkulin,  
*ADQQ* =  $\frac{1}{100}$ -proz. Lecithin.

IV. *ADRR* = 6-proz. *luetischer Leberextrakt*,  
*ADNN* = 50-proz. Bouillon,  
*ADSS* = 5-proz. Hefepreßsaft,  
*ADHH* = 75-proz. Speichel.

gesetzte komplizierte Erscheinung trat auf bei Magnesiumchlorid, Natriumbikarbonat und Trypsin.

Die geprüften Stoffe lassen sich in folgendem Schema zusammenstellen:

- a) Stoffe, welche mit normalen Tierseren die Erscheinung der Ablenkung geben.
1. Säuren: Glykokoll (Aminosäure), Oelsäure, Phosphorsäure, (Salzsäure).

2. Fermente: Hefepreßsaft, Lab, Malzextrakt, Pepsin, Speichel.
3. Kofermente und Fermentstimulatoren: Bouillon, erhitzter Lab, Leberextrakt, Lecithin, Milch (erhitzt), erhitztes Pepsin, Pepton, Tuberkulin, (erhitzter Hefepreßsaft).
4. Verschiedenes: (Agar).

b) Stoffe, welche mit solchen Seren die Erscheinung der Ablenkung nicht geben.

1. Alkalien: Glyzerin, ölsaures Kalium (unrein), Magnesiumchlorid (unrein), Natriumbikarbonat.
2. Fermente: Trypsin.
3. Verschiedenes: Eialbumin, Calciumchlorid, Gelatine, Stärke.

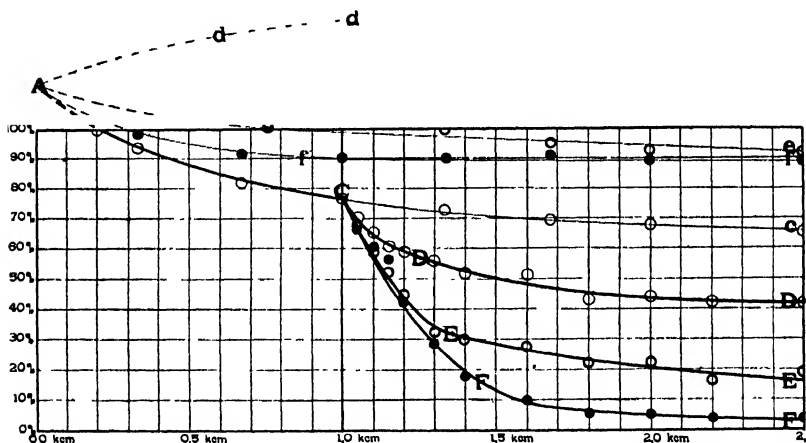


Fig. 8. Ablenkungserscheinungen bei in der Hitze inaktivierten Fermenten. In Gegenwart von 10-proz. Rinderserum gibt  $\frac{1}{10}$ -proz. Pepsin (*Aee*, *ACEE*) und  $\frac{1}{10}$ -proz. Labferment (*Aff*, *ACFF*), beides durch 30 Minuten anhaltende Erhitzung auf  $58^{\circ}$  C inaktiviert, positive Ablenkungserscheinungen. Andeutungsweise gab 5-proz. inaktivierter Hefepreßsaft (*ACDD*) die Reaktion, trotz der in ihm bei Erhitzung gebildeten auxylitischen Körper (*Add*).

Diese Versuche führten zu zwei grundlegenden Hypothesen: erstens, daß das wirksame Prinzip im Serum und Leberextrakt bei der Ablenkungserscheinung die in ihnen enthaltenen Säuren, Kofermente und Fermentstimulatoren sind, und zweitens, daß die Unterschiede, die man zwischen normalem und

luetischem Leberextrakt beobachten kann, auf quantitativ verschiedenen Mengen dieser in ihnen enthaltenen Substanzen beruhen.

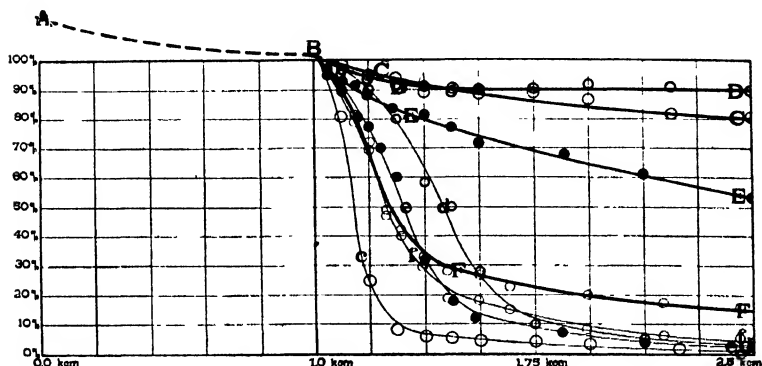


Fig. 9. Ablenkungserscheinungen bei durch Hitze inaktivierten Seren. Bei Pferde-, Rinder- und Ziegenserum wurde die ablenkende Wirkung in Gegenwart einer gleichbleibenden Menge von 1-proz. Pepton vor und nach der Zerstörung ihres Komplementes in der Hitze (56° C, 30 Minuten) geprüft:

ABcc = 5-proz. Pferdeserum,  
ABdd = 10-proz. Rinderserum,  
ABee = 10- „ Ziegenserum No. I,  
ABff = 10- „ „ „ II,  
ABCC = 5-proz. inaktiviertes Pferdeserum,

ABDD = 10-proz. inaktiviertes Rinderserum,  
ABEE = 10-proz. inaktiviertes Ziegenserum No. I,  
ABFF = 10-proz. inaktiviertes Ziegenserum No. II.

Ziege No. I war bereits über ein Jahr lang in den Institutsstallungen und früher schon zu anderen Impf- und Injektionsversuchen verwendet worden. — Ziege No. II war ein normales, kürzlich erst vom Lande aufgekauft Tier<sup>1)</sup>. Daraus, daß das Serum der Ziege No. II die ablenkende Wirkung auch nach vollständiger Zerstörung seines Komplementes in der Hitze nahezu unverändert beibehält, erhellt, daß bei dem Ablenkungsvorgang das Vorhandensein oder Fehlen des Komplementes in einem fremden Serum keine wesentliche Rolle spielt.

Ueber die Herkunft dieser abnormen Fermente inluetischen Leberextrakten ließ sich irgend ein Anhalt nicht gewinnen. Es läßt sich vermuten, daß dieselben entweder von einer Anhäufung von Kernmaterialien in kongenital veränderten Lebern

1) Für gütige Erlaubnis zur Benutzung des Serums von diesem Tier bin ich dem Institutsassistenten, Herrn Dr. Michaelis, zu Dank verpflichtet.

(zellige Infiltration usw.) oder von Zelldegenerationen herkommen<sup>1)</sup>.

Man könnte vielleicht einwenden, daß auf Grund der beschriebenen Versuche allgemeine Hypothesen nicht dürften aufgestellt werden, weil diese Seren nicht durch Hitze inaktiviert wären. Indessen zeigt eine Serie von Kontrollversuchen (Fig. 9), daß solche Seren [Ziege<sup>2)</sup>] ihre ablenkenden Eigenschaften so gut wie unverändert beibehalten, auch nachdem das Komplement auf diese Weise vollständig zerstört ist. Das beweist, daß die Erscheinung der Ablenkung bei einem gegebenen Serum von dem Vorhandensein oder Fehlen seines Komplementes unabhängig ist, ausgenommen natürlich bei Seren, die Kaninchenambozeptoren zu aktivieren imstande sind.

### 3. Ablenkungs- und Pseudoablenkungserscheinungen.

Wenn man die eben mitgeteilten Versuchsergebnisse durchmustert, kann man feststellen, daß die meisten eine Ablenkung aufweisenden Stoffe auch antilytische Eigenschaften besitzen. Deshalb schien es nicht unmöglich, daß, wenn man die verschiedenen Stadien des Ablenkungsprozesses quantitativ untersucht, man die Natur der Erscheinung näher würde erkennen können. Wenn eine gewisse Menge Pferdeserum die Wirkung einer vollen hämolytischen Dosis von 100 auf 80 Proz. herabsetzen kann, so müssen wir wissen, was für eine Wirksamkeit eine gegebene Menge Labferment gegenüber einer 80-proz. hämolytischen Dosis hat, ehe wir auf die Natur der Reaktion zwischen Pferdeserum und dem genannten Stoffe Schlüsse ziehen können. Es ist denkbar, daß eine Ablenkungserscheinung eintreten könnte; denn wenn auch das Labferment außerstande ist, bei einer vollen hämolytischen Dosis eine deutliche

---

1) Man darf nicht vergessen, daß man bei einer Gegenüberstellung von kongenital luetischen und normalen Lebern Organe vergleicht, die aus ganz verschiedenen zelligen Elementen aufgebaut sind. Während bei dem normalen Organ die Hauptmasse aus hochdifferenzierten Parenchymzellen besteht, setzt sich ein beträchtlicher Teil der luetischen Leber aus Zellen bindegewebiger oder endothelialer Natur zusammen. Es ist zu bedauern, daß bei der Herstellung von Extrakten nicht jedesmal zugleich eine sorgfältige histologische Untersuchung des verwendeten Organs stattfindet.

2) Die ablenkenden Eigenschaften des Pferde- oder Rinderserums werden durch Erhitzen so gut wie ganz zerstört.

Änderung der Wirkung herbeizuführen, so kann es doch die Wirkung einer Teildosis (80 Proz.) erheblich herabsetzen.

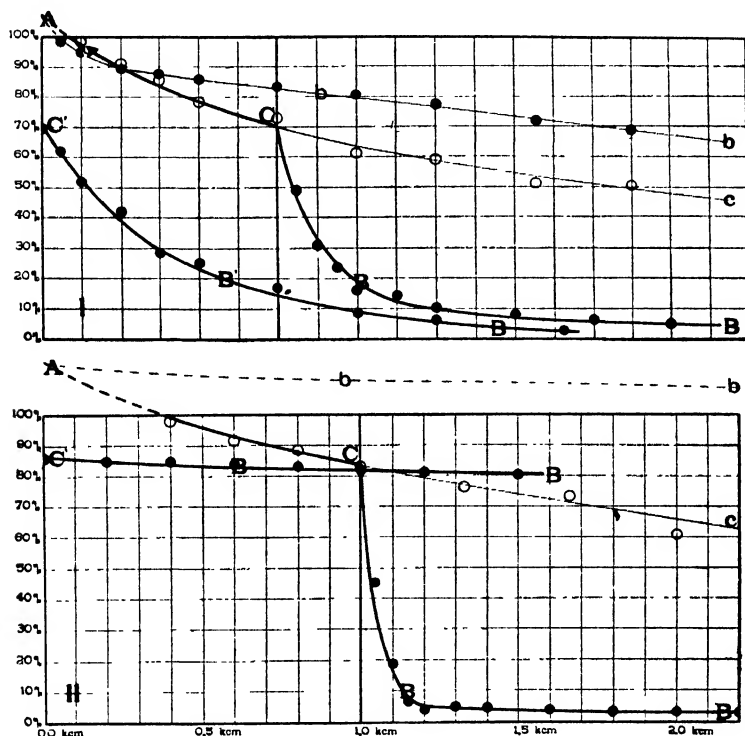


Fig. 10. Die Pseudoablenkungserscheinungen. *ACBB* (I) zeigt die hämolysenhemmende Wirkung von  $\frac{1}{3}$ -proz. Labferment, wenn solches in Gegenwart von 5-proz. Pferdeserum einwirkt. *Abb* stellt die schwache Wirkung, die dieses Ferment für sich allein einer vollen hämolysierenden Dosis gegenüber hat, dar, und *C'B'B'* seine starke Wirkung gegenüber einer geringeren (70-proz.) hämolysierenden Dosis. Der ähnliche Verlauf der beiden Kurven bei *CBB* und *C'B'B'* legt die Vermutung nahe, daß der Vorgang bei Pferdeserum und Labferment eigentlich eine Pseudoablenkung, eine Summationserscheinung zwischen zwei unabhängigen antilysischen Körpern ist.

*ACBB* (II) zeigt eine ähnliche hämolysenhemmende Wirkung bei gewöhnlicher Nährbouillon, die in Gegenwart von 5-proz. Ziegenserum einwirkt. Da die Bouillon für sich allein eine hämolysenhemmende Wirkung weder einer vollen noch einer geringeren (85-proz.) hämolysierenden Dosis gegenüber hat (*Abb*, *C'B'B'*), wird man diese Reaktion nicht als eine Summationserscheinung auffassen können.

Bei der oben angeführten Reaktion zwischen Pferdeserum und Labferment ist dies in der Tat der Fall (Fig. 10 I); Lab-

ferment ist tatsächlich imstande, dieselbe Hemmung der Hämolyse bei der reduzierten Dosis zu veranlassen, wie es die

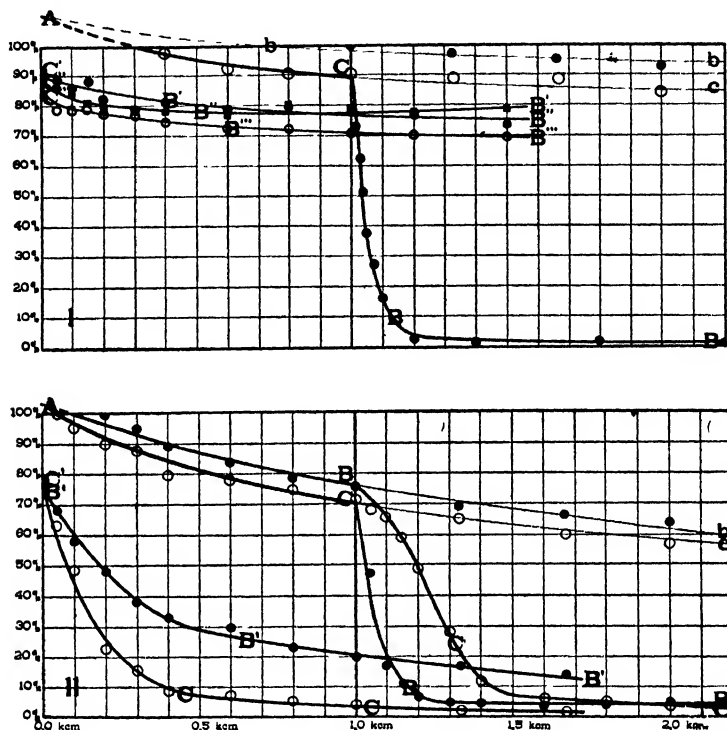


Fig. 11. Die Pseudoablenkungserscheinung. Bei I ist die Reaktion zwischen 2-proz. Pepton und 10-proz. Rinderserum in derselben Weise wie bei Fig. 10 dargestellt; es ist ersichtlich, daß das Pepton für sich allein einem geringeren (90-proz.) hämolytischen System gegenüber eine antihämolytische Wirkung nicht hat. Diese Wirkung wurde bei drei verschiedenen geringwertigen Hämolsinen geprüft.  $C'B'B'$  zeigt dieselbe Probe mit einem geringeren Hämolsin, das die normale Komplementmenge und eine geringere Menge Ambozeptoren hat;  $C''B''B''$  hat normale Ambozeptoren und verringertes Komplement, während bei  $C'''B'''B'''$  Ambozeptor wie Komplement reduziert sind.

Bei II ist eine ähnliche Reaktion zwischen 10-proz. Rinderserum und 50-proz. Tuberkulin dargestellt; Rinderserum ( $BCC$ ) sowohl als auch Tuberkulin ( $C'B'B'$ ) haben, wie ersichtlich, deutliche antihämolytische Wirkung gegenüber einem schwächeren (70-proz.) hämolytischen System. Man wird also annehmen müssen, daß die Wirkung zwischen Rinderserum und Tuberkulin eine Pseudoablenkungs-, eine Summationserscheinung ist.

entsprechende Mischung einer vollen hämolytischen Dosis bei Pferdeserum tut. Die Hämolsenhemmung muß also wahr-

scheinlich als eine Summationserscheinung zweier unabhängiger antilytischer Körper aufgefaßt werden.

Im Gegensatz zu dieser Feststellung ist in derselben Figur (II) die Analyse der Wirkung von Ziegenserum und Bouillon wiedergegeben, wobei es sich zeigt, daß die Bouillon auf ein solches hämolytisches Teilsystem eine selbständige antilytische Wirkung nicht besitzt. Die Wirkung des Ziegen-serums und der Bouillon kann infolgedessen als eine Summationserscheinung nicht aufgefaßt, sondern muß auf gänzlich verschiedene Ursachen zurückgeführt werden.

Einen ähnlichen Gegensatz zeigt Fig. 11; hier stellt sich die Wirkung von Pferdeserum und Pepton als eine echte Ablenkungserscheinung dar (I), während die zwischen Rinder-serum und Tuberkulin wahrscheinlich eine Summations-erscheinung ist.

Diese Versuche führen zu der Hypothese, daß wahr-scheinlich zwei verschiedene Faktoren bei den Erscheinungen mitwirken, die man gewöhnlich zu der Wassermanschen Reaktion rechnet. Der erste Faktor ist eine Summationswirkung von

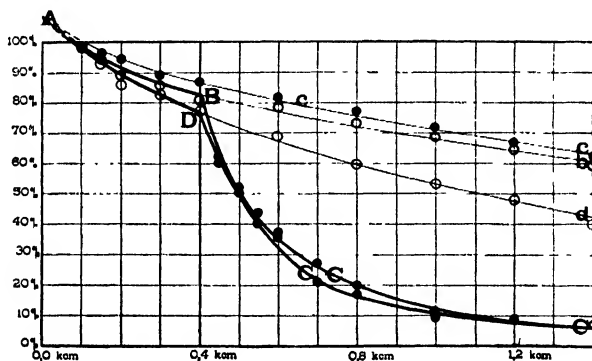


Fig. 12. Tuberkulin. Bei *ABCC* und *ADCC* stellt sich die hämolytischenhemmende Wirkung des Serums eines Rindes dar, welches wiederholt mit menschlichem Tuberkulin injiziert worden war, und zwar zeigt *Acc* die Wirkung des Serums für sich allein, *ADd* in Gegenwart von menschlichem und *ABb* in Gegenwart von Rindertuberkulin. Da sich diese Reaktion bei den vorhergehenden Versuchen (Fig. 11) als eine Pseudoablenkungs-erscheinung herausgestellt hat, so kann man aus diesem Versuche irgendwelche Schlüsse nicht ziehen, weder bezüglich Vorhandenseins oder Fehlens tuberkulöser Antikörper im Serum des Rindes, noch bezüglich der biologischen Beziehungen zwischen menschlichen und bovinen Tuberkelbacillen.

selbständigen antilytischen Körpern, der andere Faktor sind durch gegenseitige Einwirkung auf chemischem Wege entstehende antilytische Körper. Einer dieser beiden Faktoren kann bei der Reaktion die Hauptrolle spielen, so daß die Erscheinung einer Ablenkung auftritt, sei es infolge reiner Summation, sei es infolge einer rein chemischen synthetischen Wirkung.

Dieses Ergebnis ist nicht nur von theoretischem, sondern auch von praktischem Interesse, zumal der einen der oben erwähnten Reaktionen, der zwischen Rinderserum und Tuberkulin, ein diagnostischer Wert beigemessen wird. Da diese Reaktion vermutlich nur eine einfache Summationserscheinung ist, die dem Wassermannschen Phänomen nur oberflächlich ähnelt, sind solche diagnostischen Reaktionen nicht beweisend. So kann man aus dem in Fig. 12 dargestellten Versuch, bei welchem die Reaktion des Serums eines öfters mit menschlichem Tuberkulin injizierten Rindes gegenüber menschlichem und bovinem Tuberkulin geprüft ist, irgendwelche Schlüsse bezüglich Vorhandenseins oder Fehlens von tuberkulösen Antikörpern oder bezüglich des Verhältnisses zwischen den verschiedenen Tuberkelbacillenstämmen nicht ziehen.

#### 4. Autolyse des Komplementes.

Ein Faktor, der möglicherweise eine große Rolle bei der Wassermannschen Reaktion spielt, ist die spontane Degeneration oder Autolyse des Meerschweinchenkomplementes. Es wurde vermutet, daß man Anhaltspunkte bezüglich einer eventuellen Bedeutung dieses Faktors würde gewinnen können, wenn man die Degeneration des Meerschweinchenkomplementes unter der Einwirkung schwacher Säuren und Alkalien untersuchte. Um diese Degeneration zu bestimmen, ließ man verschiedene Mengen sehr schwacher  $\left(\frac{n}{100}\right)$  Säuren und Alkalien auf gleiche Mengen Meerschweinchenkomplement einwirken, und zwar unter Verhältnissen und in Zeiträumen, wie bei der Ablenkungsperiode der Wassermannschen Reaktion. Dann wurden die Säuren und Alkalien neutralisiert und das übrig bleibende Komplement durch anschließende Hämolyse bestimmt.

Das Ergebnis eines solchen Versuches zeigt Fig. 13 (I) bei *FEDABC*. Man kann daraus ersehen, daß die spontane Degeneration durch das Vorhandensein schwacher Säuren er-

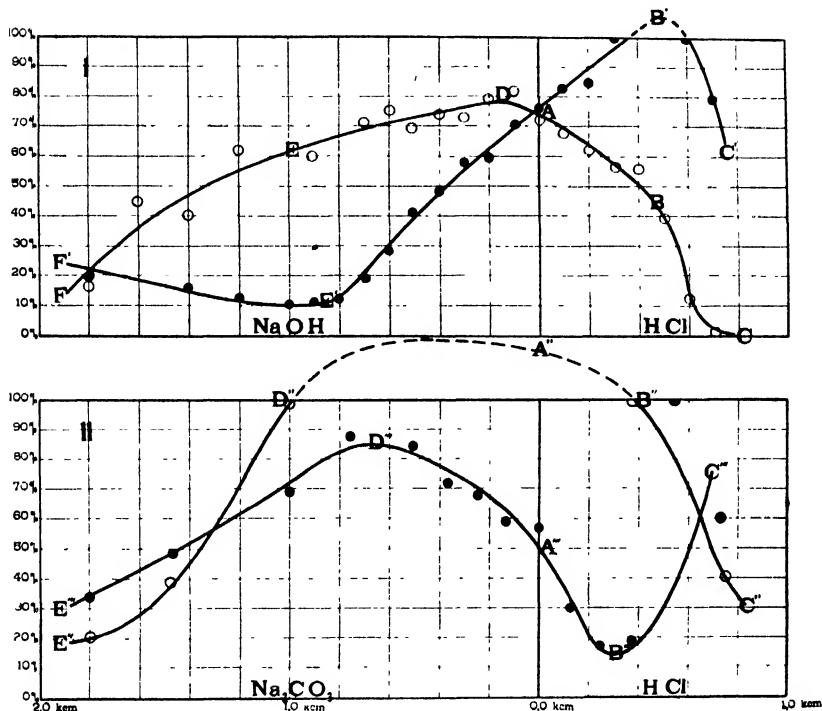


Fig. 13. Autolyse des Komplementes. *FEDABC* (I) zeigt die autolytische Zerstörung von Meerschweinchenkomplement in Gegenwart von  $\frac{n}{100}$  NaOH und  $\frac{n}{100}$  HCl; die Autolyse hat bei einem einstündigen Aufenthalt im 37° Brutschrank wie bei der Ablenkungsperiode der Wassermannschen Reaktion stattgefunden; dann wurde die Säure bezw. das Alkali neutralisiert und der Rest des Komplementes durch Zusatz sensibilisierter Blutkörperchen bestimmt. Bei *F'E'A'B'C'* (II) ist die Wirkung derselben Säure und desselben Alkali auf den eigentlichen hämolytischen Prozeß (natürlich gemeinsam mit ihrer entsprechenden Einwirkung auf die Autolyse) dargestellt, und bei *E''D''A''B''C''* (II) eine Verbindung der vorangehenden autolytischen Wirkung und der folgenden Hämolyse. *E'''D'''A'''B'''C'''* (II) zeigt diese selbe gemeinsame Wirkung bei Zusatz von 1,0 ccm 5-proz. Pferdeserum.

heblich beschleunigt wird; vollständige Zerstörung des Komplementes tritt ein, sobald bei den Versuchsbedingungen der

Wassermannschen Reaktion der Säuregrad  $\frac{n}{500}$  erreicht. In geringeren Mengen zugesetzte Alkalien hingegen hemmen diese normale Autolyse; bei Zusatz größerer Mengen ( $\frac{n}{150}$ ) indessen können sie zu einer vollständigen Zerstörung des Komplementes führen.

Im Zusammenhang damit war es ferner nötig, die direkte Wirkung desselben Körpers auf den Vorgang der Hämolyse zu bestimmen, da bei der Wassermannschen Reaktion die die Autolyse während der Ablenkungsperiode der Reaktion beeinflussenden Körper auch bei der folgenden hämolytischen Prüfung anwesend sind. Um diese zweite Wirkung zu bestimmen, wurden Säuren und Alkalien zu gleichen Komplementmengen zugesetzt und der Wirkung dieser Mischung sofort sensibilisierte Blutkörperchen unterworfen.

Das Ergebnis eines solchen Versuches zeigt  $F'E'A'B'C'$  (I). Daraus kann man ersehen, daß die Blutkörperchenlyse auch durch Säuren beschleunigt wird; die Hämolyse steigt rasch, bis die Säure eine Konzentration von  $\frac{n}{1000}$  erreicht. Dann findet ein steiler Abfall statt, vermutlich infolge Zerstörung des Komplementes. Alkalien hingegen bewirken von Anfang an einen Abfall der hämolytischen Wirkung; eine vollständige Hemmung der Hämolyse tritt ein, sobald die Konzentration  $\frac{n}{1250}$  erreicht, wobei nur eine geringe oder gar keine Zerstörung von Komplement eintritt.

Eine kombinierte Wirkung — erst autolytische Wirkung, dann blutkörperchenlösende Wirkung — sieht man bei  $E''D''A''B''C''$  (II); hieraus erhellt, daß schwache Säuren in dieser doppelten Rolle die Hämolyse auf den Nullpunkt herabdrücken, sobald die Konzentration  $\frac{n}{600}$  erreicht; auch Alkalien setzen in dieser doppelten Rolle die Hämolyse herab, indes nur, wenn sie in weit stärkerer Konzentration vorhanden sind.

Prüft man diese doppelte Wirkung in Gegenwart von Pferdeserum [ $E''D''A''B''C''$  (II)], so zeigt sich, daß Pferde-

serum die Fähigkeit hat, den Einfluß von Säuren erheblich zu vermehren und entsprechend den von Alkalien herabzusetzen. Bei Anwesenheit von Pferdeserum beobachtet man eine tatsächlich so gut wie völlige Hemmung der Hämolyse bei  $\frac{n}{1500}$  HCl.

Auf Grund dieser Versuche lassen sich folgende drei Hypothesen aufstellen: Erstens, daß im Meerschweinchen-serum ein verhältnismäßig starkes proteolytisches Ferment vorhanden ist. Dasselbe ist inaktiv in alkalischen Medien, schwach aktiv in neutralen und stark aktiv in sauren Medien, und findet sich in solchen Mengen vor, daß man es für die völlige Zerstörung des Komplementes in dem Zeitraum und unter den Verhältnissen der Wassermannschen Reaktion verantwortlich machen kann. Zweitens, daß im Leberextrakt und in Seren, welche eine positive Wassermannsche Reaktion geben, gewisse Stoffe, vermutlich Säuren, Kofermente und Fermentstimulatoren, vorhanden sind, welche die Wirkung dieses Ferments erheblich vergrößern können. Und drittens, daß die sogenannte Ablenkungserscheinung bei Syphilis ein Vorgang ist, der zum mindesten teilweise auf die durch dieses Ferment unter geeigneten Bedingungen der Reaktion des Mediums herbeigeführte Zerstörung des Meerschweinchenkomplementes zurückzuführen ist.

##### 5. Physikalisch-chemische Gesetze.

Es ist möglich, daß bei der Erscheinung der Ablenkung feste mathematische Beziehungen zwischen den reagierenden Stoffen obwalten. Darauf, daß dies in der Tat der Fall ist, weist Fig. 14 hin. Indessen genügen die Resultate noch nicht, um mehr als allgemeine Vermutungen über die eventuelle Natur solcher Beziehungen zu äußern. Offenbar ist eine Minimaldosis einer jeden regierenden Substanz für eine vollständige Ablenkung notwendig; eine völlige Hemmung ist nicht möglich, wenn eine von den Substanzen in geringerer

Menge vorhanden ist. Und ein Ueberschuß einer dieser Substanzen über die Minimaldosis hinaus spielt offenbar bei der Reaktion keine Rolle. Dies spricht für die Bildung bestimmter

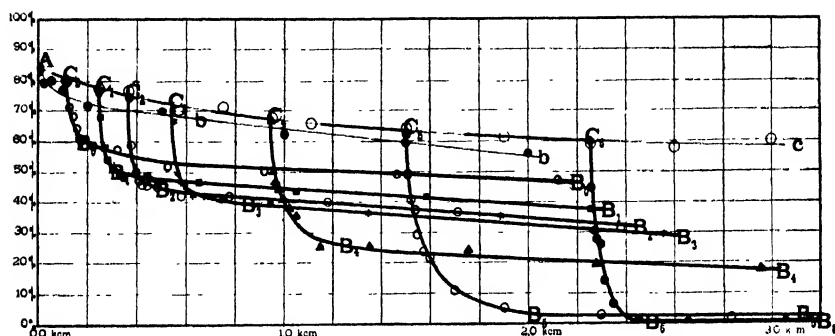


Fig. 14. Quantitative Beziehungen.  $AC_0B_0B_0$ ,  $AC_1B_1B_1$ ,  $AC_2B_2B_2$  usw. zeigen die hämolysenhemmende Wirkung von 4-proz. Pepton in Gegenwart von verschiedenen Mengen von 13-proz. Rinderserum. Dieser Versuch zeigt, 1) daß eine gewisse minimale Menge Pepton sowohl wie Rinderserum zu einer völligen Hemmung der Hämolysen nötig ist, und 2) daß bei dem Pepton eine Ueberschreitung dieser unteren Grenzen nach oben hin ohne besondere antihämolysische Wirkung ist. Parallelversuche, bei denen das Serum zu verschiedenen Mengen von Pepton zugesetzt wurde, zeigen in ähnlicher Weise die Wirkungslosigkeit des überflüssigen Serums.

chemischer Verbindungen; indessen hat sich ein Anhalt dafür, ob dieselben ihrer Natur nach Aufbauprodukte oder hydrolytische Spaltungsprodukte sind, nicht finden lassen.

## 6. Schlüsse und allgemeine Gesichtspunkte.

Wenn auch die vorstehende, unterbrochene Arbeit zur Aufstellung von mehr als Versuchshypothesen nicht genügt, so reicht sie doch aus, um den folgenden Standpunkt festzulegen; von diesem Standpunkt aus ist die Wassermannsche Reaktion nicht als eine einzelne, isoliert dastehende Erscheinung aufzufassen, sondern als ein Glied aus einer langen Kette gleicher und entgegengesetzter Erscheinungen. Infolgedessen muß die landläufige Erklärung, daß die Erscheinung auf einer Komplementbindung durch spezifische Antikörper beruhe, nur im Sinne einer lediglich heuristischen Hypothese verstanden werden, denn:

1) lassen sich deutliche Erscheinungen hervorrufen bei Versuchen, in denen die Anwesenheit solcher Antikörper ausgeschlossen ist, und

2) gibt die Hypothese, daß die Erscheinung auf einer Zerstörung von Komplement durch proteolytische Enzyme beruht, eine ebenso haltbare Erklärung.

Von diesem Gesichtspunkte aus gewinnt die offenbare Spezifität der Reaktion ein ganz besonderes Ansehen, und weist auf das Vorhandensein früher nicht beachteter Faktoren auf dem Gebiete der Immunitätschemie hin. Die Wassermannsche Entdeckung ist also nicht als eine Ausdehnung unserer Kenntnisse auf dem gegenwärtig bekannten Gebiete der cytologischen Immunität zu betrachten, sondern als eine Pionierarbeit auf einem neuerschlossenen Gebiet der Serochemie, deren weiterer Ausbau vielleicht sehr wertvolle Aufschlüsse über die Vorgänge des Stoffwechsels geben und diagnostische und therapeutische Erfolge von weittragender Bedeutung zeitigen wird <sup>1)</sup>).

1) Bemerkung von A. Wassermann: Ich möchte nicht verfehlen, zu bemerken, daß die vorstehende Arbeit, wenn auch auf der mir unterstellten Abteilung des Institutes für Infektionskrankheiten ausgeführt, in ihren Schlußfolgerungen die persönlichen Ansichten des verehrten Herrn Verfassers wiedergibt. Diese schienen mir aber doch so anregend und interessant, daß ich trotz meiner in vielen Punkten abweichenden Stellung zu dem Gegenstand mit großem Vergnügen Herrn Kollegen Manwaring Gelegenheit gab, diese mühevollen Untersuchungen in meinem Laboratorium in so exakter Weise auszuführen. — Ich möchte indessen den Eindruck vermeiden, daß auch ich meinen von Anfang an eingenommenen Standpunkt betreffs des Vorhandenseins einer spezifischen Antigen-Komponente und demgemäß eines spezifischen „Antikörpers“ bei der Luesreaktion aufgegeben hätte.



# **Über die Lichtextinktion, das Gasbindungsvermögen und den Eisengehalt des menschlichen Blutfarbstoffs in normalen und krankhaften Zuständen.<sup>1)</sup>**

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## **Einleitung.**

Eine der wichtigsten Fragen für das Verständnis der Blutkrankheiten, Anämie und Polycythämie, ist die nach dem Sauerstoffbindungsvermögen des Blutes. Bevor man jedoch damit beginnt, Versuche über die Gasbindung des pathologischen Blutes anzustellen und aus diesen Versuchen irgend welche Schlüsse zu ziehen, muß man darüber im klaren sein, wie die Verhältnisse bei normalem Blut und bei dem aus normalem Blut dargestellten krystallinischen Farbstoff liegen. Schon hierüber findet man in der Literatur die widersprechendsten Angaben. In einer Reihe von Arbeiten, die sich über einen Zeitraum von 30 Jahren erstrecken, hat Hüfner auf Grund sorgfältiger Versuche den Satz aufgestellt, daß die Lichtextinktion, die Sauerstoffaufnahme und der Eisengehalt des im normalen Blut enthaltenen Hämoglobins zu einander in konstanter Beziehung stehende Größen seien. Den absoluten Wert dieser Größen hat er durch verbesserte Methodik immer genauer zu ermitteln versucht. Gegen diese einfache und wahrscheinliche Anschauung, nach der das Oxyhämoglobin ein einheitliches, scharf charakterisiertes, chemi-

<sup>1)</sup> Die Arbeit wurde mit Unterstützung vom Rockefeller Institute for Medical Research (New-York) ausgeführt.

sches Individuum sei, hat Bohr im Jahre 1891 eine Reihe von Bedenken erhoben. Auf Grund eigener Versuche und unter Heranziehung der älteren einander widersprechenden Angaben über die Menge des von 1 g Hämoglobin gebundenen Sauerstoffs sowie über den Eisengehalt des Hämoglobins, hat Bohr<sup>1)</sup> sich berechtigt gefühlt, «die Aufmerksamkeit auf das Vorkommen verschiedener Modifikationen des Oxyhämoglobins hinzuleiten». Diese Modifikationen sollten sich dadurch charakterisieren, «daß die von ihnen gebundene Sauerstoffmenge für jede von verschiedener Größe ist». Im Gegensatz zu der herrschenden Ansicht über das Hämoglobin weist er nach, «daß das auf gewöhnliche Weise aus dem Blute dargestellte kristallinische Hämoglobin eine Mischung verschiedener einander verwandter Stoffe ist und daß die von demselben per Gramm gebundene Sauerstoffmenge nicht konstant ist». (S. 76.) Über die konstitutiven Unterschiede seiner verschiedenen Hämoglobine sagt Bohr: «Die starken Variationen in dem Eisengehalt und dem Molekulargewicht berühren selbstverständlich nicht notwendig den sauerstoffbindenden, eisenhaltigen, gefärbten Kern des Hämoglobinmoleküls, sondern lassen sich ebensowohl herleiten aus den Variationen des anderen, nicht gefärbten Teiles, des zweiten der beiden Teile, aus denen, wie wir es uns vorstellen, das Hämoglobinmolekül zusammengesetzt ist.» (S. 92.) Bohr geht aber noch viel weiter, indem er, auf eine tabellarische Zusammenstellung seiner Werte der Lichtextinktion, der Sauerstoffaufnahme und des Eisengehaltes verschiedener, durch Zusatz von Äther gewonnenen Hämoglobinpräparate hinweisend, sagt: «Wovon nun die Veränderungen des Eisengehaltes und des Molekulargewichtes herrühren mögen, so geht es ferner aus der Tabelle mit Sicherheit hervor, daß die Zusammensetzung des gefärbten Kernes im Hämoglobin ebenso wenig konstant sein kann.» Dieser letzte merkwürdige Schluß steht in schroffstem Widerspruch zu den Untersuchungen von Nencki und seinen Schülern, von Hüfner, Küster und Pregl.

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<sup>1)</sup> Die Zitate sind den Arbeiten Bohrs im Skandinavischen Archiv f. Physiologie, Bd. III, 1892, wörtlich entnommen.

Wir wollen uns an dieser Stelle nicht weiter auf den Streit zwischen Hüfner und Bohr einlassen, uns vielmehr darauf beschränken, gegen diesen Satz Bohrs einige neuere Arbeiten aus dem Tübinger Institut anzuführen. Direkte Beweise für die Konstanz des Verhältnisses von Eisengehalt zu Gasbindungsvermögen sind von Hüfner und Küster<sup>1)</sup> und von Pregl<sup>2)</sup> für das Kohlenoxyhämochromogen erbracht worden. Hervorzuheben ist, daß bei diesen Versuchen die in Frage stehende Beziehung durch gravimetrische und volumetrische Methode unter Ausschluß spektrophotometrischer Methoden ermittelt worden ist. Die Resultate dieser Versuche stimmen mit den gasanalytischen Versuchen Hüfners, welchen spektrophotometrische Bestimmungen als Grundlage dienten, vollkommen überein. Damit fällt der von andern öfters erhobene Einwand gegen die quantitative Bestimmung der Hämoglobinderivate mittels des Spektrophotometers.

Die Resultate einer größeren Versuchsreihe über kristallisiertes Hämoglobin faßt Bohr in folgenden Worten zusammen: «Ein aus verschiedenen Blutproben dargestelltes Hämoglobin ist ein Produkt, welches, von der Lage der Absorptionsstreifen abgesehen, in gar keinem wesentlichen Charakterzug konstant ist.» (S. 94.)

Nachdem Bohr den Begriff der Hämoglobine mit verschiedenem Sauerstoffbindungsvermögen aufgestellt hat, läßt er Versuche mit frischem, defibriniertem Hundeblood folgen, aus denen er dieselben Schlüsse bezüglich Eisengehalt, Lichtextinktion und Sauerstoffaufnahme zieht, wie er sie schon für den kristallisierten Farbstoff gezogen hat. Diese Schlüsse über «den in den Blutkörperchen noch eingeschlossenen Farbstoff» sind in Bohrs eigenen Worten folgende: «Das Verhältnis zwischen dem Eisengehalt und der Lichtextinktion des Blutes ist in Wirklichkeit kein konstantes» (S. 103), und «die Menge Sauerstoff, welche im Blute einer Einheit absorbierten Lichtes entspricht, ist ganz so veränderlich als die Menge, welche per

<sup>1)</sup> Hüfner und Küster, Arch. f. Anat. u. Physiol., physiol. Abtl., Suppl., 1904, S. 387.

<sup>2)</sup> F. Pregl, Diese Zeitschrift, Bd. XLIV, 1905, S. 173.

Gramm Eisen gebunden ist.» (S. 107.) Diese Zitate lassen, glaube ich, die Ansichten Bohrs über diesen Gegenstand klar erkennen. Ich habe mich mit Absicht auf die diesen Gegenstand berührend experimentellen Arbeiten Bohrs beschränkt, da er sich auf sie in seiner Zusammenstellung, «Blutgase und respiratorischer Stoffwechsel» im Handbuch der Physiologie von Nagel wiederholt beruft, ohne für seine von Hüfner<sup>1)</sup> widerlegten Behauptungen neue Beweise zu bringen.

Diese auf Tierversuche sich stützenden Anschauungen Bohrs sind von mehreren Seiten aufgenommen worden. Die Annahme Bohrs von der Existenz von Hämoglobinen mit verschiedenem Sauerstoffbindungsvermögen bringt es nahe, ähnliche Betrachtungen auf die Menschenpathologie zu übertragen und in der Bildung eines Gemisches von vorwiegend sauerstoffreicheren bzw. sauerstoffärmeren Hämoglobinen bei Anämie und Polycythämie die Möglichkeit eines Kompensationsvorganges zu suchen. Versuche, die dies beweisen sollen, liegen tatsächlich vor. So findet Mohr<sup>2)</sup> bei anämisch gemachten Hunden ein erhöhtes Sauerstoffbindungsvermögen des Blutes und Lommel<sup>3)</sup> ein um fast die Hälfte des normalen Wertes erniedrigtes Sauerstoffbindungsvermögen des Menschenblutes bei Polycythämie. Diesen Arbeiten steht die größere an Menschenblut ausgeführte Untersuchung von Kraus, Kossler und Scholz<sup>4)</sup> gegenüber. Aus ihren Versuchen (Hämoglobin mit dem Glandschen Spektrophotometer, Sauerstoff mit der Blutgaspumpe bestimmt) schließen die genannten Autoren, sie hätten keine Tatsache gefunden, welche den Satz, daß im Blut von gesunden und anämischen Menschen einerlei Hämoglobin vorhanden ist, umstoßen würde. Dieselben Forscher wiesen nach, daß die Sauerstoffkapazität des defibrinierten Blutes anämischer Menschen mit dem auf spektrophotometrischem Wege ermittelten Hämoglobin-

<sup>1)</sup> Hüfner, Arch. f. Anat. u. Physiol., physiol. Abtl., 1894, S. 130.

<sup>2)</sup> Mohr, Zeitschr. f. exp. Pathol. u. Therapie, Bd. II, S. 435.

<sup>3)</sup> Lommel, Deutsch. Arch. f. klin. Med., Bd. LXXXVII, S. 329, Bd. XCII, S. 83.

<sup>4)</sup> Kraus, Kossler und Scholz, Arch. f. exp. Pathol. u. Pharmacol., Bd. XLII.

gehalt annähernd parallel abnimmt. Bohr<sup>1)</sup> glaubt die Resultate dieser Autoren anders deuten zu müssen und sieht in den teilweise allerdings ziemlich stark schwankenden Werten nicht etwa methodische Fehler, sondern eine Stütze seiner Anschauungen über die inkonstante Zusammensetzung des Blutfarbstoffs. In neuerer Zeit hat Morawitz<sup>2)</sup> mit der Haldane-Barcroft'schen Ferricyanidmethode der Bestimmung der Sauerstoffkapazität und mit der kolorimetrischen Bestimmung des Hämoglobins eine Parallelität der beiden Werte beim normalen Menschen, bei Anämie und Polycythämie gefunden; die Werte sind indessen keine absoluten.

Die Übersicht über das umfangreiche Versuchsmaterial ist noch weiter erschwert worden durch die neuerdings aufgestellte Behauptung, daß das frische Blut vieler gesunder Tiere methämoglobinhaltig und daß darin der Grund für die verschiedenen Werte der Sauerstoffbindung zu suchen sei. Durch diese Annahme versucht H. Aron<sup>3)</sup> die Differenzen zwischen den Resultaten Hüfners und Bohrs aufzuklären. Bohr habe keine Konstanz für das Verhältnis zwischen Sauerstoffaufnahme, Lichtextinktion und Eisengehalt gefunden, meint Aron, weil er in einer Gegend des Spektrums gemessen habe, in der das Vorhandensein von Methämoglobin sich am deutlichsten bemerkbar macht, und weil er alle untersuchten Blutproben zur Berechnung des Verhältnisses von Lichtextinktion und Sauerstoffkapazität verwertet habe, während Hüfner sämtliche Blutproben, die nicht seine optische Konstante für Oxyhämoglobin zeigten, zu Gasversuchen nicht verwertet und auf diese Weise Konstanz erreicht habe. Die Behauptung, daß frisches Blut Methämoglobin neben Oxyhämoglobin enthalte, stützt sich hauptsächlich auf eine Reihe von stark schwankenden Messungen mit dem Hüfnerschen Spektrophotometer.

Es sei hier nebenbei bemerkt, daß in Blutproben, die bei richtiger Aufstellung des Hüfnerschen Apparates die gleichen

<sup>1)</sup> Bohr, Nagels Handbuch d. Physiol., Bd. I, S. 98.

<sup>2)</sup> Morawitz und Röhm, Deutsch. Arch. f. klin. Med., Bd. XCIV, S. 529.

<sup>3)</sup> H. Aron, Biochem. Zeitschr., Bd. III, S. 10.

spektrophotometrischen Werte wie die Aronschen aufweisen, Methämoglobin in Mengen, die sich leicht spektroskopisch nachweisen lassen, vorhanden ist. Bis jetzt hat jedoch noch niemand behauptet, den Methämoglobinstreifen im normalen Blut gesehen zu haben.

Aus diesen sich widerstreitenden Literaturangaben sieht man, wie sehr es nötig ist, darüber Klarheit zu schaffen, wie die Verhältnisse tatsächlich liegen. Bis jetzt liegt weder für normales Blut noch für Blut bei verschiedenen Krankheiten eine größere Untersuchung über Lichtextinktion, Gasbindungsvermögen und Eisengehalt vor. An der Hand einer solchen mit guter Methodik durchgeführten Untersuchung muß es sich leicht entscheiden lassen, ob bestimmte Beziehungen, die auf eine konstante Zusammensetzung des Hämoglobinmoleküls hinweisen, herrschen, oder ob Eisengehalt, Lichtextinktion und Sauerstoffaufnahme in durchaus regelloser Beziehung zu einander stehende Größen sind. Die vorliegende Arbeit gliedert sich daher naturgemäß in drei Teile:

- I. Spektrophotometrischer Teil.
- II. Gasanalytischer Teil.
- III. Eisenbestimmungen.

## **I. Spektrophotometrischer Teil.**

Geschichtliches und Theoretisches. Die Prinzipien der Spektrophotometrie sind schon von vielen Autoren, namentlich Vierordt, Hüfner, Lambling, Cherbuliez und Saint-Martin, elementar und zusammenfassend dargestellt worden. Trotzdem ist eine kurze Klarlegung der Prinzipien an dieser Stelle notwendig, da neuere Arbeiten<sup>1)</sup> auf diesem Gebiete eine ziemliche Verwirrung in die an sich recht einfachen Ver-

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<sup>1)</sup> H. Aron und F. Müller, Arch. f. Anat. u. Physiol., physiol. Abtl., Suppl., S. 109.

H. Aron, Biochem. Zeitschr., Bd. III, S. 1.

F. Müller, Handbuch d. Biochemie, herausgegeben von Oppenheimer, Bd. I, S. 654.

J. Plesch, Hämodynam. Studien, Berlin 1909 (Aug. Hirschwald), S. 53.

hältnisse gebracht haben. Es sei deshalb in aller Kürze an die physikalischen Gesetze der Lichtabsorption und der meßbaren Abschwächung des Lichtes durch Polarisationsvorrichtungen sowie an die Anwendung dieser Gesetze im allgemeinen und im speziellen Fall des Blutfarbstoffs erinnert.

Die Absorption des Lichtes folgt folgenden Gesetzen.

I. Die Menge des durchgelassenen Lichtes ist der des auffallenden proportional.

Hierbei ist von Lichtverlusten durch Reflexion abgesehen. Wenn man sich das auffallende Licht aus parallelen Strahlen homogenen Lichtes denkt, so ist

II. Die Menge des durchgelassenen Lichtes der Schichtdicke des absorbierenden Körpers umgekehrt proportional, oder anders ausgedrückt, gleiche Schichtdicken eines Körpers absorbieren immer den gleichen Bruchteil des auffallenden Lichtes.

Es sei  $J$  die Intensität des auffallenden,  $J'$  die des durchgelassenen Lichtes, dann ist die Proportionalität durch  $\frac{J'}{J} = \alpha$  gegeben, wobei  $\alpha$  eine von der Natur des Körpers und von der Wellenlänge des Lichtes abhängige Konstante ist;  $\alpha$  ist, wie leicht ersichtlich, der Bruch, mit dem man die auffallende Lichtmenge multiplizieren muß, um die Menge des durchgelassenen Lichtes zu erfahren, es ist also  $J' = J\alpha$ . Wenn nun  $\alpha$  der Bruchteil des durchgelassenen Lichtes für eine Einheit der Schichtdicke ist, dann wird  $J'$ , da gleiche Schichtdicken einer Substanz immer gleiche Mengen Licht absorbieren bzw. immer den gleichen Bruchteil hindurchlassen, nach Passieren von 2, 3 oder  $d$  hintereinander stehenden Schichteinheiten  $J\alpha^2$ ,  $J\alpha^3$ , . . . . .  $J\alpha^d$  sein.

Die Beziehung

$$J' = J\alpha^d$$

ist der einfachste Ausdruck der Absorptionsgesetze. Die Gleichung macht die Proportionalität der durchgelassenen und der auffallenden Lichtmenge und das geometrische Verhältnis der Lichtschwächung zu der Schichtdicke anschaulich.  $\alpha$  ist ein Zahlenfaktor, der für jeden absorbierenden Körper charakte-

ristisch ist und dessen Wert sich mit der Wellenlänge ändert. Man rechnet in der Photometrie farbiger Substanzen nicht direkt mit der Menge des absorbierten Lichtes, sondern mit einem Koeffizienten, wie  $\alpha$ , der angibt, den wievielten Teil der auffallenden Lichtmenge die absorbierende Substanz noch durchläßt. Im folgenden haben wir es ausschließlich mit Körpern zu tun, bei denen eine auswählende Absorption stattfindet, d. h. mit Körpern, die bei einzelnen Wellenlängen viel stärker absorbieren als bei den übrigen, und die daher farbig erscheinen. Die Anwendung der eben besprochenen Prinzipien zur quantitativen Bestimmungsmethode für farbige Substanzen ergibt sich von selbst. Man kann sich eine beliebig dicke Schicht einer Lösung eines farbigen Körpers aus vielen kleineren gleichgroßen hintereinander stehenden Schichten zusammengesetzt denken. In einem homogenen System sind gleichviel Moleküle in gleichdicken Schichten enthalten. Je mehr absorbierende Moleküle sich dem Gang der Lichtstrahlen in den Weg stellen, desto größer wird, *ceteris paribus*, der Lichtverlust sein. Ob eine ganz bestimmte Anzahl Moleküle auf einer dünneren oder dickeren Schicht verteilt ist, ist für den Effekt — die Größe der Absorption — ganz gleichgültig und deshalb können Schichtdicke und Konzentration miteinander vertauscht werden. Das Absorptionsvermögen eines Körpers ist also der Konzentration bzw. Schichtdicke proportional. Der Beweis hierfür ist zuerst von Beer<sup>1)</sup> erbracht worden.

In der Spektrophotometrie wird  $\alpha$  als Maß der Lichtextinktion nur selten gebraucht. Man bedient sich einer anderen durch Bunsen und Roscoe eingeführten und jetzt fast allgemein eingebürgerten Einheit als Maß der Lichtschwächung. Diese Einheit, der Extinktionskoeffizient, ist lediglich aus rech-

<sup>1)</sup> Beer, Poggendorffs Annalen d. Physik, Bd. LXXXVI, S. 78, 1852.

Siehe ferner: Vierordt, Die Anwendung des Spektralapparates usw., Tübingen 1873; E. Müller, Drudes Annalen d. Physik, Bd. XII, S. 767, und F. Grünbaum, ebendas., S. 1004, 1903; Hantzsch und Glover, Ber. d. Deutsch. chem. Ges., Bd. XXXIX, 4153, 1906; Hantzsch, Ber., Bd. XL, 1556, 1907, und Bd. XLI, 213 und 217, 1908; Hantzsch, Zeitschr. f. physikal. Chemie, 1908.

nerischen Gründen gewählt worden. Die Proportionalität der durchgelassenen Lichtmenge zu der auffallenden ist durch das Experiment für endliche Schichtdicken streng gültig gefunden worden. Man darf annehmen, daß die Proportionalität auch für unendlich kleine Schichtdicken gilt. «Geht man von dieser Annahme aus und nennt man  $J$  die Lichtintensität vor der Durchstrahlung,  $J'$  die Lichtintensität nach Durchstrahlung einer Körperschicht von der Dicke  $d$ , und nennt man endlich  $\frac{1}{\epsilon}$  die Dicke der Körperschicht, nach deren Durchstrahlung die Intensität der einfallenden Strahlen auf  $1/10$  herabgesunken ist, so hat man zwischen der durchgelassenen Lichtmenge  $J$  und der Dicke der durchstrahlten Schicht die Gleichung:

$$J' = J \cdot 10^{-\epsilon d}$$

und daraus

$$\epsilon = \frac{1}{d} \log \left( \frac{J}{J'} \right). \text{ } ^1)$$

Wir werden von jetzt ab nur  $\epsilon$  als Maß der Lichtschwächung gebrauchen.

Drücken wir die Lichtextinktion mit  $\epsilon$  aus, dann läßt sich die Proportionalität zwischen dem Lichtextinktionsvermögen eines Körpers und der Konzentration auf die folgende Form bringen:

$$\epsilon_1 : c_1 = \epsilon_2 : c_2 \quad \text{oder}$$

$$\frac{\epsilon_1}{c_1} = \frac{\epsilon_2}{c_2} = \frac{\epsilon_3}{c_3} = \dots = \text{konst.}$$

wobei  $\epsilon_1$ ,  $\epsilon_2$  und  $\epsilon_3$  die zu den Konzentrationen  $c_1$ ,  $c_2$  und  $c_3$  zugehörigen Extinktionskoeffizienten bedeuten. Drücken wir die Konzentration als Grammoleküle im Liter aus, dann erhalten wir das spezifische Lichtextinktionsvermögen eines Körpers. Dieses wird als Molekularextinktion,  $E$ , bezeichnet und ist bei gegebener Wellenlänge eine spezifische Eigenschaft des betreffenden Körpers.

Die Proportionalität kann wiederum ebensogut durch

<sup>1)</sup> Bunsen und Roscoe, Photochemische Untersuchungen, Pogg. Annalen d. Physik, Bd. CI, S. 237.

$$\frac{c_1}{\epsilon_1} = \frac{c_2}{\epsilon_2} = \frac{c_3}{\epsilon_3} = \dots = \text{konst.}$$

zum Ausdruck gebracht werden, nur hat hier natürlich die Konstante einen anderen Zahlenwert. Diese Konstante wird nach Vierordt das Absorptionsverhältnis,  $A$ , bezeichnet.

Die letztere Form,

$$\frac{c_1}{\epsilon_1} = \frac{c_2}{\epsilon_2} = \dots = A,$$

ist die Form, in der das Beersche Absorptionsgesetz am meisten angegeben wird. Wenn man das Absorptionsverhältnis einer Lösung eines farbigen Körpers ermittelt hat, so kann man aus dem Extinktionskoeffizienten die Konzentration anderer Lösungen desselben Körpers berechnen; denn da  $\frac{c}{\epsilon} = A$  ist, so ist  $c = \epsilon A$ . In diesem Fall bedeutet  $c$  immer die Bruttokonzentration.

Messung des Extinktionskoeffizienten. Aus der Beziehung  $\epsilon = \frac{1}{d} \log \frac{J}{J'}$  ersieht man, daß es zur Berechnung des Extinktionskoeffizienten nur nötig ist, das Verhältnis von  $J$  zu  $J'$  zu ermitteln, da die Schichtdicke der Lösung,  $d$ , sich sehr leicht direkt messen läßt. Die Messung des Verhältnisses der beiden Lichtintensitäten kann auf verschiedene Weise vorgenommen werden. Meistens benützt man Licht von nur einer Lichtquellé, um mittels Linsen und Prismen zwei neben- oder übereinander liegende Gesichtsfelder gleichmäßig zu beleuchten. Der Gang der Strahlen wird so angeordnet, daß in den einen Strahlengang die absorbierende Lösung gebracht werden kann, während in dem anderen irgend eine Vorrichtung zur meßbaren Abschwächung des Lichtes sich findet. Im folgenden werden wir es nur mit der Lichtschwächung durch eine Polarisationsvorrichtung zu tun haben. Fällt linear polarisiertes Licht, von der Schwingungsrichtung  $OC$  und der Amplitude  $Oc$  (Fig. 1) auf ein Nicolsches Prisma, dessen Hauptschnitt  $OA$  mit  $OC$  einen Winkel  $\alpha$  bildet, dann wird das Licht in zwei Komponenten  $Oa$  und  $Ob$  zerlegt, von denen nur die im Hauptschnitt schwingende  $Oa$  durchgelassen wird.

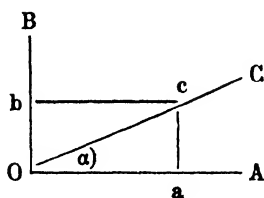


Fig. 1.

Da die Intensität gleich dem Quadrat der Amplitude ist, so ist die Intensität des durchgelassenen Strahles gleich  $\cos^2 \alpha$ . Sind OC und OA parallel, so geht alles Licht durch; steht OC senkrecht auf OA, dann geht kein Licht durch. Wenn man zwei Nicols anwendet, so kann man mit dem einen linear polarisiertes Licht erzeugen und mit dem anderen das polarisierte Licht meßbar schwächen. Man trifft die Anordnung so, daß in dem einen Strahlengang die absorbierende Lösung und in dem anderen der polarisierende Nicol sich finden und daß beide Strahlengänge dann den zweiten Nicol durchsetzen; durch Drehung dieses letzteren stellt man auf gleiche Helligkeit der beiden Gesichtsfelder ein. Man dreht von der Stellung aus, bei der die Hauptschnitte der beiden Nicols einander parallel sind, d. h., in der Stellung, wo alles Licht von dem ersten Nicol durch den zweiten ungeschwächt hindurchgeht. Sind in dieser Stellung der Nicolschen Prismen beide Gesichtsfelder vor Einschaltung der absorbierenden Lösung gleich, dann ist nach Einschalten der Lösung die Intensität des durchgelassenen Lichtes gleich  $\cos^2$  desjenigen Winkels, um welchen man die Hauptschnitte der beiden Nicols gegeneinander drehen mußte, um gleiche Helligkeit der beiden Gesichtsfelder zu erzielen. Nehmen wir die auffallende Lichtintensität zu 1 an und arbeiten wir ausschließlich mit einer Schichtdicke von 1 cm (wie es bei dem Hüfnerschen Apparat üblich ist), so erhält die Definitionsgleichung des Extinktionskoeffizienten die folgende einfache Form,

$$\epsilon = -\log \cos^2 \alpha = -2 \log \cos \alpha.$$

Andere Vorrichtungen zur meßbaren Schwächung des Lichtes außer den Polarisationsvorrichtungen sind laterale Spaltverengerung oder die quadratische Blende und der rotierende Sektor.

Die Absorptionsgesetze gelten nur für homogenes Licht. Um dieses zu erzeugen, wird das Licht durch ein dispergierendes Prisma zerlegt und die Extinktion bei bestimmten Wellenlängen gemessen.

### Anwendung auf das Blut.

1. Quantitative Bestimmung des Oxyhämoglobins im Blut. Da der Extinktionskoeffizient sich auf 1% Genauigkeit mit einem guten Spektrophotometer bestimmen läßt, und da die Herstellung einer Lösung von bekanntem Gehalt an mehrmals umkrystallisiertem Hämoglobin keine allzugroße Schwierigkeit bietet,<sup>1)</sup> kann man das Absorptionsverhältnis A für den betreffenden Apparat bei gegebener Wellenlänge endgültig bestimmen. Bei den Bestimmungen des Oxyhämoglobingehaltes des Blutes ist es nur nötig, den Extinktionskoeffizienten  $\epsilon$  einer genau verdünnten Lösung mit A zu multiplizieren, um gemäß der Beziehung,  $c = \epsilon \cdot A$ , natürlich unter Berücksichtigung der Verdünnung die Konzentration zu erfahren. Das Absorptionsverhältnis wird nach Hüfner an zwei Stellen im Spektrum des Oxyhämoglobins bestimmt; bei dem Minimum der Absorption im Gelb, einer mittleren Wellenlänge von 560  $\mu\mu$  entsprechend, und in der Mitte des zweiten Streifens im Grün bei 538  $\mu\mu$ . Mit einem richtig aufgestellten Hüfnerschen Apparat läßt sich das Oxyhämoglobin im Blut auf 2,5% Genauigkeit bestimmen.

2. Charakterisierung eines Körpers. Mit Rücksicht auf das große Molekulargewicht des Hämoglobins und die damit verbundene Unsicherheit bei seiner Bestimmung hat man bis jetzt die Molekularextinktion nicht als spezifische Eigenschaft des Blutfarbstoffs herangezogen. Vielmehr hat Hüfner zur

<sup>1)</sup> Man ist allerdings gezwungen, zur Herstellung der Lösung einen Umweg einzuschlagen. Da das Oxyhämoglobin nicht vollständig getrocknet werden kann, ohne sich teilweise in Methämoglobin umzuwandeln, stellt man die Lösung in der Weise dar, daß man den feuchten Krystallbrei nach möglichst vollständiger Befreiung von der Mutterlauge durch Zentrifugieren in Wasser löst und in einem Teil der Lösung den Trockenrückstand bestimmt.

Charakterisierung des Hämoglobins von einem anderen sehr einfachen optischen Verfahren Gebrauch gemacht. Wie oben erwähnt wurde, wird das Absorptionsverhältnis für das Oxyhämoglobin immer für zwei Regionen im Spektrum ermittelt. Jedes dieser Absorptionsverhältnisse stellt eine Konstante dar, und die Konzentrationsberechnung muß gleich ausfallen, gleichgültig welches A und der dazugehörige Extinktionskoeffizient der Berechnung zugrunde gelegt werden. Es ist also,

$$c = A\epsilon = A'\epsilon',$$

wobei im speziellen Fall der Blutfarbstoffe A das Absorptionsverhältnis für die Region  $554\ \mu\mu - 565\ \mu\mu$  und  $\epsilon$  der in dieser Gegend gemessene Extinktionskoeffizient sind, und A' das Absorptionsverhältnis für  $531,5\ \mu\mu - 542\ \mu\mu$  und  $\epsilon'$  der Extinktionskoeffizient in dieser Region. Daraus erhalten wir  $\frac{A}{A'} = \frac{\epsilon'}{\epsilon}$ .

Für eine beliebige andere Konzentration  $c_2$  haben wir ebenfalls  $A\epsilon_2 = A'\epsilon'_2 = c_2$  und wieder daraus  $\frac{A}{A'} = \frac{\epsilon'_2}{\epsilon_2}$ ; folglich ist  $\frac{\epsilon'}{\epsilon} = \frac{\epsilon'_2}{\epsilon_2}$ . Man ersieht hieraus, daß der Quotient zweier

an verschiedenen Stellen im Spektrum gemessenen Extinktionskoeffizienten eine Konstante ist, die unabhängig von der Konzentration und charakteristisch für den betreffenden Farbstoff ist. Für die verschiedenen Blutfarbstoffe ist der Zahlenwert des Quotienten verschieden und eben deshalb ein sehr wichtiges Hilfsmittel zu ihrer Identifizierung. Unter den Hüfnerschen Versuchsbedingungen (Breite des Eintrittsspalt  $1/40$  mm, Spektralgegend wie oben angegeben, Rauchglaskeil auf 0,5) ist der Wert des Quotienten z. B. für Oxyhämoglobin 1,58, für Methämoglobin 1,19, Kohlenoxydhämoglobin 1,10 und für das reduzierte Hämoglobin 0,76.

3. Gleichzeitige quantitative Bestimmung zweier Farbstoffe im Blut. Das Prinzip beruht darauf, daß, wenn man zwei Farbstofflösungen, die nicht chemisch miteinander reagieren, mischt, die Extinktion des Gemisches die algebraische Summe der Extinktionen der Komponenten darstellt. Wenn man bei 2 verschiedenen Wellenlängen mißt, am besten dort,

wo die Extinktionen der beiden Farbstoffe möglichst voneinander verschieden sind, kann man nach Vierordt aus den Extinktionskoeffizienten des Gemisches und den Absorptionsverhältnissen beider Farbstoffe zwei Gleichungen mit zwei Unbekannten aufstellen, und nach der einen oder der anderen Unbekannten auflösen. Damit ist die Möglichkeit gegeben, aus einer Lichtextinktionsmessung in zwei Regionen im Spektrum den absoluten Gehalt einer Lösung an zwei Farbstoffen zu berechnen. Voraussetzung dabei ist die Kenntnis von den vier Absorptionsverhältnissen.

Bei physiologischen Arbeiten kommt es nicht so häufig darauf an, die absolute Menge der beiden Farbstoffe als vielmehr den prozentualen Gehalt der Lösung an beiden Körpern zu erfahren. Für die Hämoglobinderivate hat Hüfner das Mischungsverhältnis zwischen 2 Derivaten aus Änderung des Quotienten  $\frac{\epsilon'}{\epsilon}$  abgeleitet. Hat man, beispielsweise, ein Gemisch von Oxyhämoglobin,  $\frac{\epsilon'}{\epsilon} = 1,58$ , und reduziertem Hämoglobin,  $\frac{\epsilon'}{\epsilon} = 0,76$ , dann muß der Quotient des Gemisches einen Wert haben, der zwischen diesen beiden Zahlen liegt. Man kann mit Hilfe einer Interpolationsformel die Prozente Oxyhämoglobin oder Hämoglobin für alle zwischenliegende Werte von  $\frac{\epsilon'}{\epsilon}$  berechnen. Tabellen mit diesen Werten für Gemische von Oxyhämoglobin und reduziertem Hämoglobin, Oxyhämoglobin und Kohlenoxydhämoglobin und Oxyhämoglobin und Methämoglobin sind schon von Hüfner<sup>1)</sup> angegeben worden.

#### Experimentelles.

Nach den Absorptionsgesetzen stellt der Quotient  $\frac{\epsilon'}{\epsilon}$  für chemisch einheitliche Stoffe eine Konstante dar. Er ist daher für das Studium des Blutfarbstoffes außerordentlich wichtig. Wenn das normale mit Sauerstoff gesättigte Blut nur einen

<sup>1)</sup> Hüfner, Arch. f. Anat. u. Physiol., physiol. Abtl., 1899, S. 39.

Farbstoff enthält und dieser eine einheitliche Zusammensetzung hat, dann muß  $\frac{\epsilon'}{\epsilon}$  bei gleich bleibenden Versuchsbedingungen immer denselben Wert haben, und zwar sowohl für das frische Blut, als auch für das aus dem Blut krystallinisch dargestellte Oxyhämoglobin. Konstanz der Beziehung  $\frac{\epsilon'}{\epsilon}$  für das krystallinische Oxyhämoglobin wie auch für das Blut verschiedener Tiere ist von Hüfner und seinen Schülern, von Lambing, Cherbuliez und Saint-Martin, gefunden worden. Dagegen ist von Bohr und seinen Mitarbeitern eine Inkonzanz des Lichtextinktionsvermögens des Blutfarbstoffes behauptet worden. Ferner haben die Untersucher im Zuntz'schen Laboratorium, insbesondere H. Aron, F. Müller und J. Plesch, nicht nur Inkonzanz für  $\frac{\epsilon'}{\epsilon}$  gefunden, sondern sie haben auch eine wesentlich niedrige Zahl als Durchschnittswert dieses Quotienten angegeben, als Hüfner und andere ihn gefunden haben. Ohne zunächst auf eine Erklärung dieser widersprechenden Resultate einzugehen, möge zuerst eine Reihe von eigenen Bestimmungen des Wertes für  $\frac{\epsilon'}{\epsilon}$  an normalem Blut verschiedener Tiere und an Lösungen von Oxyhämoglobin, das aus einigen dieser Blutproben dargestellt wurde, sowie einige Bestimmungen an normalem und pathologischem Menschenblut und Menschenhämoglobin angeführt werden. Hier sei ausdrücklich betont, daß die Zahlen keine ausgesuchten sind, sondern daß sie die laufenden täglichen Untersuchungen von Blutproben, die nachher zu Gasversuchen, Hämoglobindarstellung usw. verwendet wurden, darstellen. (Tab. I.)

Aus dieser Tabelle geht mit Sicherheit hervor, daß der Wert von  $\frac{\epsilon'}{\epsilon}$  bei gleich gehaltenen Versuchsbedingungen ein durchaus konstanter ist. Diese Konstanz gilt nicht nur für das frische normale Blut vom Menschen und von Tieren, sondern auch für das Menschenblut bei den wichtigsten Blutkrankheiten, wie Polycythämie, Chlorose, perniziöse Anämie und Skorbut.

Tabelle I.

$\varphi$  und  $\varphi'$  bedeuten die Drehungswinkel des analysierenden Nicols in den Regionen  $564,6 \mu\mu$  —  $556,1 \mu\mu$  und  $542 \mu\mu$  —  $533,5 \mu\mu$ ;  $\epsilon$  und  $\epsilon'$  sind die daraus berechneten Extinktionskoeffizienten.

## Rinderblut.

$\epsilon$	$\epsilon'$	$\frac{\epsilon'}{\epsilon}$	$\varphi$ Grad	$\varphi'$ Grad
0,563	0,883	1,57	58,45	68,80
0,943	1,51	1,60	70,27	79,83
0,642	1,04	1,62	61,49	72,40
0,848	1,33	1,57	67,49	77,48
0,587	0,931	1,59	59,41	69,99
0,673	1,07	1,59	62,55	73,08
0,607	0,974	1,60	60,17	70,98
0,737	1,17	1,59	64,64	74,99
0,498	0,783	1,57	55,69	66,05
0,522	0,827	1,58	56,76	67,30
0,403	0,642	1,59	51,06	61,47
0,560	0,884	1,58	58,33	68,82
0,622	0,988	1,59	60,75	71,29
0,655	1,04	1,59	61,95	72,39
0,685	1,07	1,56	62,97	73,11
0,527	0,833	1,58	56,96	67,47
0,549	0,860	1,57	57,90	68,19
0,587	0,920	1,57	59,43	69,72
0,469	0,741	1,58	54,35	64,78
0,506	0,795	1,57	56,04	66,39
0,493	0,782	1,59	55,47	66,02
0,589	0,928	1,58	59,51	69,90
0,619	0,980	1,58	60,65	71,13
0,680	1,07	1,57	62,80	72,93
0,429	0,682	1,59	52,40	62,86
0,488	0,772	1,58	55,26	65,71
0,709	1,12	1,58	63,77	73,95
0,731	1,14	1,56	64,47	74,44
0,465	0,732	1,57	54,17	64,51
0,627	0,999	1,59	60,94	71,55

Tabelle I.  
Rinderblut.

Fortsetzung.

$\epsilon$	$\epsilon'$	$\frac{\epsilon'}{\epsilon}$	$\varphi$ Grad	$\varphi'$ Grad
0,509	0,802	1,58	56,17	66,60
0,633	0,996	1,57	61,16	71,48
0,607	0,948	1,56	60,18	70,39
0,550	0,865	1,57	57,95	68,33
0,737	1,17	1,59	64,67	74,87
0,514	0,807	1,57	56,41	66,74
0,671	1,06	1,58	62,49	72,81
0,576	0,901	1,56	58,98	69,25
0,596	0,937	1,57	59,76	70,11
0,444	0,701	1,58	53,13	63,49
0,640	0,990	1,55	61,41	71,35
0,625	0,992	1,59	60,84	71,38
0,697	1,10	1,58	63,38	73,70
0,663	1,05	1,58	62,20	72,64

	$\epsilon$	$\epsilon'$	$\frac{\epsilon'}{\epsilon}$	$\varphi$ Grad	$\varphi$ Grad
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## Blut von verschiedenen Tieren.

Huhn . . . . .	0,569	0,918	1,61	58,72	69,67
Ratte . . . . .	0,888	1,45	1,63	68,92	79,09
Kaninchen . . . . .	0,481	0,759	1,57	54,91	65,36
Pferd . . . . .	0,637	1,00	1,57	61,28	71,64

## Blut von gesunden und kranken Menschen.

Gesunder . . . . .	0,805	1,25	1,55	66,68	76,34
	0,885	1,38	1,56	68,84	78,17
Gicht . . . . .	0,560	0,887	1,58	58,36	68,88
Nephritis . . . . .	0,645	1,02	1,58	61,58	72,00
	0,764	1,22	1,59	65,48	75,82
Herzklappenfehler . . . .	0,697	1,08	1,55	63,38	73,20
	0,479	0,759	1,58	54,80	65,32
Hemiplegie . . . . .	0,655	1,03	1,57	61,92	72,16
	0,761	1,19	1,56	65,40	75,20

Tabelle I.

Fortsetzung.

	ε	ε'	$\frac{\epsilon'}{\epsilon}$	φ Grad	φ Grad
Blut von gesunden und kranken Menschen.					
Polycythämie I. . . . .	0,639	1,01	1,58	61,36	71,88
	0,770	1,23	1,60	65,66	76,00
Polycythämie II. . . . .	0,617	0,974	1,58	60,56	70,98
	0,702	1,12	1,59	63,54	73,96
Polycythämie III. . . . .	0,644	1,01	1,57	61,56	71,84
	0,708	1,13	1,60	63,74	74,10
Polycythämie IV. . . . .	0,605	0,952	1,57	60,12	70,48
	0,696	1,11	1,59	63,32	73,82
Chlorose I. . . . .	0,489	0,773	1,58	55,30	65,74
	0,767	1,22	1,59	65,56	75,80
Chlorose II. . . . .	0,930	1,49	1,60	69,96	79,60
	0,721	1,13	1,57	64,14	74,10
Chlorose III. . . . .	0,897	1,43	1,59	69,14	78,90
Perniciöse Anämie (H) . .	0,608	0,945	1,56	60,18	70,32
	0,802	1,27	1,58	66,60	76,56
Perniciöse Anämie (M) . .	0,705	1,11	1,57	63,64	73,80
	0,884	1,40	1,58	68,80	78,44
Skorbut . . . . .	0,647	1,01	1,56	61,64	71,84
	0,787	1,26	1,60	66,16	76,50
Pseudoleukämie . . . . .	0,697	1,12	1,59	63,38	73,94
	0,867	1,36	1,57	68,36	77,96
Krystallisiertes Oxyhämoglobin.					
Aus Rinderblut I. . . . .	0,640	1,01	1,58	61,37	71,69
	0,797	1,27	1,59	66,45	76,59
Aus Rinderblut II. . . . .	0,442	0,700	1,58	53,04	63,48
	0,521	0,818	1,57	56,70	67,05
Aus Rinderblut III . . . .	0,654	1,05	1,61	61,90	72,52
	0,908	1,41	1,55	69,42	78,68
Aus Pferdeblut . . . . .	0,620	0,968	1,56	60,66	70,84
	0,718	1,13	1,57	64,06	74,17
Aus Menschenblut . . . .	0,553	0,863	1,56	58,06	68,26
	0,630	0,995	1,58	61,05	71,45
	0,758	1,18	1,56	65,30	75,16
	0,806	1,25	1,55	66,72	76,30

Abgerundete Mittelwerte für  $\frac{\epsilon'}{\epsilon}$  bei verschiedenen Blut- und Hämoglobinarten.

Rinderblut (25 Tiere)	1,58
Menschenblut (16 Fälle)	1,58
Rinderhämoglobin (3 Darstellungen)	1,58
Menschenhämoglobulin (1 Darstellung)	1,56
Pferdehämogloblin (1 Darstellung)	1,57

Sonstige einzelne Bestimmungen:

Hühnerblut	1,61
Rattenblut	1,63
Kaninchenblut	1,57
Pferdeblut	1,57

Gesamtmittel . . . 1,58

(Aus 88 Bestimmungen an 50 Blut- und Hämoglobinproben.)

Noch einmal sei betont, daß die Rinderblutproben 25 konsequente Lieferungen vom Tübinger Schlachthof sind; die Werte stellen daher keine Auslese dar.

Der Zahlenwert von  $\frac{\epsilon'}{\epsilon}$  bei der genannten Versuchsanordnung

(Breite des Eintrittsspalt  $\frac{1}{40}$  mm, Rauchglaskeil auf 0,0, herausgeschnittenes Spektralgebiet  $564,6 \mu\mu$ — $556,1 \mu\mu$  und  $542 \mu\mu$ — $533,5 \mu\mu$  ist für Blut von verschiedenen Tieren, für normales und pathologisches Menschenblut sowie auch für krystallisiertes Menschen-, Pferde- und Rinderoxyhämoglobin 1,58.

Erst nachdem man sich überzeugt hatte, daß das Oxyhämoglobin sich jedenfalls seinem Spektralverhalten nach als ein einheitlicher Körper verhält, hatte es einen Sinn, seine quantitative Bestimmung auf optischem Weg vorzunehmen. Hierzu brauchten wir noch die Kenntnis des Absorptionsverhältnisses A. Obwohl diese Größe schon von Hüfner und anderen mehrmals bestimmt worden ist, war es aus zwei Gründen trotzdem notwendig, dieses Verhältnis von neuem zu bestimmen.

1. Das Absorptionsverhältnis ist von dem relativen Wert von  $\epsilon$  abhängig; der relative Wert von  $\epsilon$  hängt wiederum von dem Lichtverlust im Apparat ab, da  $\epsilon$  durch das Verhältnis  $J/J'$  bestimmt ist. Es ist deshalb möglich, daß man mit zwei verschiedenen Apparaten an ein und derselben Lösung verschiedene Werte für  $\epsilon$  finden kann. Auch A muß in diesem

Fall für die beiden Apparate verschiedene Werte haben. So erklärt es sich, daß man mit verschiedenen Apparaten übereinstimmende Resultate bei quantitativen Bestimmungen erhält, während A nicht notwendig den gleichen Wert bei jedem Apparat zu haben braucht.

So fand v. Noorden<sup>1)</sup> mit dem ersten Hüfnerschen Spektrophotometer das Absorptionsverhältnis des Hundehämoglobins bei einem Spektralbezirk von  $569,4 \mu\mu$ — $556,3 \mu\mu$  zu  $132 \cdot 10^{-5}$ ; Otto<sup>2)</sup> mit demselben Apparat in Tübingen das Absorptionsverhältnis des Schweinehämoglobins im gleichen Spektralgebiet  $135 \cdot 10^{-5}$ , in Christiania<sup>3)</sup> aber mit einem anderen Hüfnerschen Apparat das Absorptionsverhältnis des Hundehämoglobins ebenfalls bei  $569,4 \mu\mu$ — $556,3 \mu\mu$  zu  $188 \cdot 10^{-5}$  und mit dem Vierordtschen Spektrophotometer zu  $144 \cdot 10^{-5}$ . Hüfner<sup>4)</sup> selber hat das Absorptionsverhältnis mit seinem ersten Apparat im genannten Spektralgebiet zu  $147 \cdot 10^{-5}$  und mit seinem neuen Apparat in einem etwas engeren Spektralgebiet zu  $207 \cdot 10^{-5}$  bestimmt. Mit Ausnahme des letzteren Wertes sind alle anderen Werte ohne Angabe über die Breite des Eintrittspaltes und den Stand des Rauchglaskeiles mitgeteilt. Wie wir sehen werden, sind diese zwei Faktoren von großem Einfluß auf den Wert des Extinktionskoeffizienten und somit auch auf A.

2. Da unsere Versuche zum Endziel hatten, das Blut gesunder und kranker Menschen zu untersuchen, so brauchten wir natürlich das Absorptionsverhältnis für menschliches Oxyhämoglobin. Dieses war bisher nicht bekannt, mußte aber, wenn wir absolute Hämoglobinbestimmungen im Menschenblut machen wollten, bestimmt werden.

Tabelle II gibt die mit dem neuesten Hüfnerschen Spektrophotometer (1907) — dieses unterscheidet sich von den anderen Hüfnerschen Apparaten bloß dadurch, daß die Nicolschen

<sup>1)</sup> v. Noorden, Diese Zeitschrift, Bd. IV.

<sup>2)</sup> J. Otto, Diese Zeitschrift, Bd. VII.

<sup>3)</sup> J. Otto, Pflügers Archiv, Bd. XXXVI, S. 18 und 23.

<sup>4)</sup> G. Hüfner, Diese Zeitschrift, Bd. III, und Arch. f. Anat. u. Physiol., physiol. Abtl., 1894.

Prismen und die Ausführung der mechanischen Teile besser sind — bei unserer Versuchsanordnung gewonnenen Zahlen für das Absorptionsverhältnis von Menschen- und Rinderhäoglobin. Als Anhang dazu geben wir noch das Resultat einer Bestimmung des Absorptionsverhältnisses von Methämoglobin in alkalischer Lösung bei einer mittleren Wellenlänge von  $560\text{ }\mu\mu$ , in der Region, wo Methämoglobin die gleiche Lichtextinktion wie Oxyhäoglobin zeigt.

Über die Ausführung der Bestimmung ist folgendes zu bemerken. Das Rinderhäoglobin wurde im wesentlichen nach dem Verfahren von Hoppe-Seyler dargestellt; das Menschenhäoglobin wurde aus gesundem Menschenblut durch Aus-salzen mit Ammoniumsulfat krystallinisch gewonnen.

Das Rinderblut wurde durch Schlagen defibriniert, koliert und bei 2000 bis 2400 Umdrehungen in der Minute zentrifugiert. Das Serum und die Leukocytschicht wurden von dem Blutkörperchenbrei abgehebert. Der Brei von roten Blutkörperchen wurde in möglichst wenig ausgekochtem Wasser (ev. bei  $30^{\circ}$ ) gelöst. Das lackfarben gemachte Blut wurde nach Abkühlung auf  $0^{\circ}$  mit  $\frac{1}{2}$  Volumen Äther in einem Scheidetrichter kräftig geschüttelt. Nach Trennung der Schichten wurde die wässrige Blutlösung abgelassen und durch einen Strom gewaschener Luft vom Äther befreit. Zu der wässrigen Blutlösung wurden dann wieder nach Abkühlung auf  $0^{\circ}$   $\frac{1}{4}$  Volumen abgekühlten Alkohols unter fortwährendem Umschütteln gegeben. Das ganze Gemisch wurde dann in eine Kältemischung gestellt, bis die Oxyhäoglobinkrystalle sich abgeschieden hatten. Nach vollständiger Ausscheidung der Krystalle wurden sie von der Mutterlauge durch Zentrifugieren getrennt, der Krystallbrei in möglichst wenig Wasser gelöst und durch Alkoholzusatz und Abkühlen wieder zum Krystallisieren gebracht. In dieser Weise wurde das Oxyhäoglobin mehrmals umkrystallisiert.

Beim Menschenblut gelang es nicht, das Häoglobin durch Alkoholzusatz und Abkühlen zum Krystallisieren zu bringen, wohl aber durch Aussalzen mit Ammoniumsulfat. Das auf diese Weise erhaltene Menschenhäoglobin wurde auf dem Filter mit kaltem Wasser gewaschen und wieder gelöst. Von der

Lösung des einmal krystallisierten Oxyhämoglobins wurden in aliquoten Teilen die Lichtextinktion, der Trockenrückstand und der  $\text{SO}_4$ -Gehalt bestimmt. Nach Abzug des prozentischen  $(\text{NH}_4)_2\text{SO}_4$ -Gehaltes von dem Trockenrückstand erhält man die Hämoglobinkonzentration der Lösung; es wurde selbstverständlich bei dieser Bestimmung nur bei Zimmertemperatur über Schwefelsäure im Vakuum getrocknet.

Tabelle II.

Das Absorptionsverhältnis von verschiedenen Hämoglobinpräparaten.

Art des Hämoglobins	Konzentration in 1 ccm	Extinktionskoeffizient bei $564,6 \mu\mu$ — $556,1 \mu\mu$	Absorptionsverhältnis	Mittelwert des Absorptionsverhältnisses
Oxyhämoglobin aus Rinderblut, einmal umkrystallisiert	$0,8164 \cdot 10^{-3}$	0,442	$1,85 \cdot 10^{-3}$	$1,87 \cdot 10^{-3}$
	$0,9716 \cdot 10^{-3}$	0,521	$1,87 \cdot 10^{-3}$	
	$1,447 \cdot 10^{-3}$	0,767	$1,90 \cdot 10^{-3}$	
Oxyhämoglobin aus Rinderblut, zweimal umkrystallisiert	$0,8648 \cdot 10^{-3}$	0,455	$1,90 \cdot 10^{-3}$	$1,87 \cdot 10^{-3}$
	$1,081 \cdot 10^{-3}$	0,590	$1,83 \cdot 10^{-3}$	
	$1,235 \cdot 10^{-3}$	0,669	$1,85 \cdot 10^{-3}$	
	$1,729 \cdot 10^{-3}$	0,908	$1,91 \cdot 10^{-3}$	
Oxyhämoglobin aus Menschenblut, erste Krystallisation	$1,055 \cdot 10^{-3}$	0,553	$1,91 \cdot 10^{-3}$	$1,89 \cdot 10^{-3}$
	$1,51 \cdot 10^{-3}$	0,806	$1,87 \cdot 10^{-3}$	
Methämoglobin aus Oxyhämoglobin vom Schwein	$1,44 \cdot 10^{-3}$	0,773	$1,86 \cdot 10^{-3}$	$1,86 \cdot 10^{-3}$

Die Extinktionskoeffizienten bei  $542 \mu\mu$ — $533,5 \mu\mu$  und das Absorptionsverhältnis für diese Region sind nicht besonders angeführt, da das letztere sich aus der Tabelle des Quotienten für die Hämoglobinpräparate und aus dem Absorptionsverhältnis für die Region  $564,6 \mu\mu$ — $556,1 \mu\mu$  ergibt. Um das Absorptionsverhältnis bei  $542 \mu\mu$ — $533,5 \mu\mu$  zu erhalten, braucht man nur das Absorptionsverhältnis für die andere Region durch

den Wert des Quotienten  $\frac{\epsilon'}{\epsilon}$  für das betreffende Präparat zu dividieren. Es ist im Mittel  $1,18 \cdot 10^{-3}$ .

Die Trockenbestimmungen wurden in Drechselschen Enten gemacht. Die Lösungen wurden zuerst im Vakuum-exsikkator über Schwefelsäure bis zur Trockene eingengt und dann bis zur Gewichtskonstanz in einer Wasserstoffatmosphäre bei  $110^\circ$  getrocknet.<sup>1)</sup>

Vergleichen wir nun unsere konstanten Werte für  $\frac{\epsilon'}{\epsilon}$  bei Blut- und Hämoglobinlösungen und die übereinstimmenden Werte des Absorptionsverhältnisses von Rinderhämoglobin verschiedener Darstellungen und von Menschenhämoglobin mit den inkonstanten Zahlen anderer, so drängt sich uns die Frage auf: Wie sind diese Verschiedenheiten zu erklären? Wir wollen zuerst alle Faktoren, die auf den Wert der beiden Extinktionskoeffizienten von Einfluß sein könnten, näher betrachten. Zunächst sei an die Konstruktion des Hüfnerschen Spektrophotometers<sup>2)</sup> kurz erinnert.

Der Apparat hat die äußere Form eines großen Spektralapparates und besteht, wie dieser, aus einem Kollimatorrohr und einem um das Dispersionsprisma um eine vertikale Achse

<sup>1)</sup> Hierbei stellte es sich im Gegensatz zu H. Aron und F. Müller (loc. cit., S. 121) heraus, daß es gleichgültig ist, ob man nur bei Zimmertemperatur über Schwefelsäure oder bei  $110^\circ$  im Wasserstoffstrom trocknet. 1,9899 g feuchten Krystallbreies wogen bis zur Gewichtskonstanz im Vakuumexsikkator über Schwefelsäure getrocknet 0,9790 g, nach 4stündigem Erhitzen in einer Wasserstoffatmosphäre bei  $110^\circ$  0,9780 g, nach 7stündigem Erhitzen 0,9764 g und nach 11 Stunden 0,9764 g. Ein Verlust von 0,3% nach 11 Stunden im Toluolbad; auf den Trockengehalt des Krystallbreies berechnet, erhält man statt 49,21% 49,08%. Ferner gaben 25 ccm einer Hämoglobininlösung bis zum konstanten Gewicht im Vakuum über Schwefelsäure getrocknet einen Rückstand von 1,3750 g, nach 4stündigem Erhitzen im Wasserstoffstrom bei  $110^\circ$  ein Gewicht von 1,3753 g. Das rückständige Hämoglobin war in feinen Bröckelchen über eine größere Glasfläche verteilt und nach dem Erreichen des konstanten Gewichtes über Schwefelsäure (einige Wochen) verlor es auch bei höherer Temperatur nichts mehr an Gewicht.

<sup>2)</sup> Hüfner, Zeitschr. f. physikal. Chem., Bd. III, S. 562, 1889.

drehbaren Fernrohr. An einem Sektor eines Teilkreises kann der Stand des Fernrohres in seiner Beziehung zum Prisma abgelesen werden. Die Beleuchtungsvorrichtung ist eine Auerlampe, die von einem geschwärzten Tonzylinder, die gegen den Apparat zu eine runde Öffnung trägt, umgeben ist. Eine Sammellinse ist in einem Metallansatz vor der Öffnung des Tonzylinders so angebracht, daß die am stärksten glühende Partie des Strumpfes in ihrem Brennpunkt steht. Das Ganze ist auf einer optischen Bank befestigt.

Außerdem ist vor dem Eintritts- (Kollimator-)spalt der sogenannte Albrechtsche Körper angebracht. Dieser ist ein Glashombus, der als brechendes Prisma wirkt und die Vergleichsfelder im Apparat erzeugt. Der Rhombus ist so gestellt, daß eine durch zwei seiner gegenübereinander liegenden horizontalen Kanten gedachte Ebene senkrecht auf dem Eintrittsspalt steht und diesen in zwei übereinander stehende Spalthälften trennt. Vor der unteren brechenden Fläche des Rhombus steht ein Nicolsches Prisma; vor der oberen brechenden Fläche ein verstellbarer Rauchglaskeil. Das Absorptionsgefäß ist in einem Stativ, das zwischen der Lampe und dem Apparat auf der optischen Bank verstellbar ist, eingesetzt. Das Gefäß hat planparallele Glaswände, die 11 mm von einander stehen. In das Gefäß wird ein Glasblock von 10 mm Dicke und mit planparallelen Seiten gebracht. Das Stativ für das Absorptionsgefäß ist auch in vertikaler Richtung verstellbar und wird bei der Absorptionsmessung so gestellt, daß die 1 mm-Schicht der absorbierenden Flüssigkeit und der Schulzsche Körper (das planparallele Glasblock) vor dem Nicolschen Prisma und der unteren brechenden Fläche des Glashombus zu stehen kommen, während eine 11 mm Schicht der absorbierenden Flüssigkeit vor dem Rauchglaskeil und der oberen brechenden Fläche des Rhombus steht. Bei dieser Stellung fällt der obere Rand des Schulzschen Körpers und die vordere Kante des Glashombus in eine Ebene zusammen.

Im Fernrohr ist ein zweiter, in einem Teilkreis drehbarer Nicol. Zu betonen ist, daß das Licht von beiden Strahlengängen, d. h. das aus dem ersten Nicol austretende polari-

sierte Bündel sowie das durch den Rauchglaskeil und die obere Fläche des Glashrhombus kommende Bündel natürlichen Lichtes auf den zweiten Nicol fällt.

Der Kollimator- (Eintritts-)spalt und der Okular- (Fernrohr-)spalt sind beide mit Mikrometerschrauben versehen.

Bei unserem Apparat war:

Die Breite des Eintrittsspalt  $\frac{1}{40}$  mm.

Der Stand des Rauchglaskeiles 0,0.

Die Breite des Okularspaltes entsprach einer Breite des entworfenen Spektrumbildes von  $8,5 \mu$ .

Die Kurve für die Umwertung der Skalenteile des Kreissektors in Wellenlängen wurde aus den Fraunhoferschen Linien im Sonnenspektrum von Hüfner entworfen. Die Strecke zwischen D und E wurde von einem Mathematiker geradlinig interpoliert. Die Kurve haben wir mit den Flammenspektren von Natrium, Lithium und Thallium wiederholt kontrolliert und richtig gefunden. Beleg: D-Linie der Spektralkurve  $589,3 \mu$  entspricht Teilstrich 15,5 der Sektorskala; die Linie der Natriumflamme fällt ebenfalls mit 15,5 zusammen. Die Thalliumlinie 535 entspricht 21,0—21,7 oder 21,2—21,4 Skalenteilen am Sektor, im Mittel 21,3; auf der Kurve entspricht 535  $\mu$  der Skalenteilung 21,3. Die bei  $570,8 \mu$  liegende Lithiumlinie entspricht 9,7—10,1 Skalenteilen, Mitte 9,9; auf der Kurve fällt  $570,0 \mu$  mit 9,9 der Skalenteilung zusammen. Die nahezu vollkommene Übereinstimmung ist für zwei Punkte in der interpolierten Strecke, D—E, gefunden worden, sowie für einen Punkt, der nahe der direkt bestimmten C-Linie liegt. Es war zu erwarten, daß die Übereinstimmung für die nicht interpolierten Strecken, d. h. für die Strecken, in denen der Abstand zweier Fraunhoferschen Linien durch eine gerade Linie dargestellt wird, und ganz besonders, wenn der zu kontrollierende Punkt sich nicht in der Nähe einer der direkt bestimmten Fraunhoferschen Linie befindet, nicht mehr gelten würde. So finden wir, daß die bei  $460,8 \mu$  liegende Sr-Linie der Skalenteilung 33,5 entspricht, auf der Kurve aber fallen 33,5 und  $467,0 \mu$  zusammen.

Zum richtigen Gebrauch des Hüfnerschen Spektrophoto-

meters sind außer dem Strahlengang noch folgende Punkte bei der Aufstellung zu berücksichtigen.

1. Die Breite des Okularspaltes und somit die Breite des ausgeschnittenen Spektralgebietes.

2. Die Breite des Eintrittsspalt.

3. Der Stand des Rauchglaskeiles.

4. Die Position der Beleuchtungsvorrichtung.

Wir wollen zunächst sehen, in wie weit der Extinktionskoeffizient durch eine Änderung in diesen 4 Punkten beeinflußt wird.

1. Einfluß der Okularspaltbreite auf den Wert des Extinktionskoeffizienten. Bei sämtlichen bis jetzt über die Lichtextinktion des Blutes angestellten Arbeiten sind die Werte in einem Spektralgebiet von mehreren Wellenlängen bestimmt worden, sie gelten also nicht für absolut homogenes Licht. Da die Lichtextinktion eines Körpers sich mit der Wellenlänge ändert, ist es klar, daß der Wert von  $\epsilon$ , der sich aus den verschiedenen großen Extinktionen der einzelnen Wellenlängen zusammensetzt, auch mit der Breite des herausgeschnittenen Spektralgebietes sich ändern muß. Beim Oxyhämoglobin nimmt man nun die Messung bei einem Maximum und Minimum der Extinktionen vor. Wenn man eine kurvenmäßige Darstellung der Lichtextinktion des Oxyhämoglobins — wie sie bei Cherbuliez<sup>1)</sup> oder Saint-Martin<sup>2)</sup> zu finden ist — betrachtet, so findet man zwischen den beiden Extinktionsmaxima (den beiden Streifen des Oxyhämoglobins) ein Extinktionsminimum bei  $560 \mu\mu$ ; von diesem nimmt die Extinktion ziemlich symmetrisch und steil nach beiden Seiten zu. Von dem Extinktionsmaximum bei  $542 \mu\mu$  nimmt die Extinktion auch ziemlich gleichmäßig nach beiden Seiten ab. Es ist deshalb klar, was geschieht, wenn man mit einer Spaltbreite, die mehreren Wellenlängen entspricht, bei einer mittleren Wellenlänge von  $560 \mu\mu$  und  $542 \mu\mu$  die Lichtextinktion mißt. Man mißt nicht genau die maximale und minimale Extinktion in diesen Regionen,

<sup>1)</sup> Cherbuliez, Étude spectrophotométrique du sang oxycarboné. Paris 1890.

<sup>2)</sup> Saint-Martin, Spectrophotométrie du sang. Paris 1898, S. 59.

sondern ein von beiden Seiten mit schwächeren Extinktionen begrenztes Maximum und ein Minimum mit daneben liegenden stärkeren Extinktionen. Je breiter das herausgeschnittene Spektralgebiet, desto mehr kommen die schwächeren Extinktionen zur Geltung und drücken den Wert der gesamten Extinktion in der Region des Maximums herab, und dasselbe gilt natürlich in umgekehrtem Sinne für den Wert in der Region des Minimums. Die Folge davon ist selbstverständlich, daß mit wachsender Breite des Okularspaltes  $\epsilon$  zunimmt, während  $\epsilon'$  abnimmt und  $\frac{\epsilon'}{\epsilon}$  dementsprechend kleiner wird. Folgende Messungen mit dem Spektrophotometer von König, Martens und Grünbaum illustrieren die mit wachsender Okularspaltbreite verbundene Abnahme von  $\frac{\epsilon'}{\epsilon}$ . Mit der engsten Spaltbreite,

die noch Messungen gestattet, war das Verhältnis des Extinktionskoeffizienten bei der maximalen Absorption im Grün zu dem der minimalen Absorption im Gelb, 1,68, bei einem etwas breiteren Okularspalt 1,62 und bei noch weiterem Spalt 1,56.

Es ist ferner klar, daß, wenn man nicht genau bei den mittleren Wellenlängen der maximalen und minimalen Extinktionen, sondern etwas seitwärts von diesen mißt, daß dann der Wert für  $\epsilon$  größer werden muß und der für  $\epsilon'$  kleiner. Der Einfluß der Messung auf den Wert von  $\frac{\epsilon'}{\epsilon}$ , an Stellen, die seitwärts von der maximalen oder minimalen Extinktion liegen, läßt sich aus folgender kleiner Zusammenstellung der Resultate von Cherbuliez, Hüfner und Saint-Martin ersehen.

	Spektralgebiete	Mittlere Wellenlänge	$\frac{\epsilon'}{\epsilon}$
Cherbuliez .	541 $\mu\mu$ — 536 $\mu\mu$	538,5 $\mu\mu$	1,54
	558 $\mu\mu$ — 553 $\mu\mu$	555,5 $\mu\mu$	
Hüfner . . .	542,5 $\mu\mu$ — 531,5 $\mu\mu$	537,0 $\mu\mu$	1,58
	565 $\mu\mu$ — 554 $\mu\mu$	559,5 $\mu\mu$	
Saint Martin .	549 $\mu\mu$ — 538 $\mu\mu$	543,5 $\mu\mu$	1,62
	568,3 $\mu\mu$ — 557,2 $\mu\mu$	562,5 $\mu\mu$	

2. Einfluß des Eintrittsspaltcs. Um den Einfluß der Breite des Eintrittsspaltcs auf den Extinktionskoeffizienten zu verstehen, muß man sich an die ersten Begriffe der Dispersion erinnern. Lassen wir homogenes Licht durch einen Spalt auf ein Prisma fallen, so erhalten wir hinter dem Prisma ein abgelenktes Spaltbild. Der Grad der Ablenkung hängt von der Art des Lichtes ab, er ist am geringsten für rote Strahlen und am stärksten für violette Strahlen. Lassen wir nun weißes Licht auf den Spalt fallen, dann entsteht eine kontinuierliche Reihe von nebeneinanderliegenden Spaltbildern in allen Farben von Rot bis ins Violett. Hätte der Spalt eine genügend kleine Breite, dann würde jeder Punkt des entworfenen Bildes einem Spaltbild einer einzigen Art homogenen Lichtes entsprechen. Da aber der Spalt immer eine endliche Breite hat, so greifen die Bilder übereinander. Das Spektrum wird um so unreiner, je breiter der Spalt ist. Ähnlich ist es auch mit der Abbildung des Absorptionsspektrums. Es kommt im Falle des Oxyhämoglobins mit wachsender Spaltbreite zu der maximalen Absorption bei  $542 \mu$  eine Reihe von angrenzenden schwächeren Absorptionen hinzu, die infolge ihres niedrigen Extinktionsvermögens den Wert für  $\epsilon'$  herabdrücken. Bei der minimalen Absorption bei  $560 \mu$  machen sich die angrenzenden stärkeren Absorptionen mit Erweiterung des Eintrittsspaltcs geltend und der Wert für  $\epsilon$  steigt. Demnach sinkt der Wert von  $\frac{\epsilon'}{\epsilon}$  mit wachsender Breite des Eintrittsspaltcs, wie es auch der Fall war bei wachsender Breite des Okularspaltcs. Die folgenden Werte machen den Einfluß des Eintrittsspaltcs auf den Wert von  $\epsilon$  und  $\epsilon'$  anschaulich. Es wurden wieder bei der maximalen Extinktion im Grün und bei der minimalen im Gelb mit dem Apparat von König, Martens und Grünbaum gemessen. Die Breite des Okularspaltcs war möglichst klein gewählt und blieb konstant bei allen Messungen. Eine Blutlösung und eine Lösung von frisch dargestelltem Rinderhämoglobin wurden innerhalb zwei Stunden durchgemessen.

	Breite des Eintrittsspaltcs			
	0,13 mm	0,25 mm	0,50 mm	1,00 mm
I. Lösung von Menschenblut	$\epsilon'$ 0,589	0,586	0,571	0,520
	$\epsilon$ 0,352	0,352	0,373	0,408
	$\frac{\epsilon'}{\epsilon}$			
	1,67	1,67	1,54	1,27
II. Lösung von Rinder- hämoglobin	$\epsilon'$ 0,561	0,559	0,546	0,501
	$\epsilon$ 0,332	0,335	0,350	0,391
	$\frac{\epsilon'}{\epsilon}$			
	1,68	1,67	1,56	1,28

Grünbaum<sup>1)</sup> hat auch auf die Abhängigkeit des Wertes des Extinktionskoeffizienten von der Spaltbreite beim Arbeiten mit weißem Licht als Beleuchtungsquelle aufmerksam gemacht.

3. Einfluß des Standes des Rauchglaskeiles. Da im unteren Strahlengang des Hüfnerschen Spektrophotometers zwei Nicols sind, während in dem oberen bloß einer, der analysierende ist, hat Hüfner, um etwaige Lichtverluste im unteren Strahlengang auszugleichen, einen verschiebbaren Rauchglaskeil vor der oberen Hälfte des Eintrittsspaltcs angebracht. Dieses Verfahren hat aber sehr wenig Berechtigung. Wir wissen, daß von einem Nicolschen Prisma immer die Hälfte des auffallenden natürlichen Lichtes durchgelassen wird. Ferner, daß das einem Nicol austretende linear polarisierte Licht von einem zweiten Nicol ungeschwächt durchgelassen wird, wenn die Hauptschnitte der beiden Prismen einander parallel sind. Bei dieser Stellung der Nicolschen Prismen im Hüfnerschen Apparat kommen demnach bloß zwei Reflexionen mehr für den unteren Strahlengang als weitere Quelle von Lichtverlusten in Betracht. Besteht das auffallende Licht aus annähernd parallelen Strahlen, so wird der Lichtverlust äußerst gering sein. Nun gerade in der Nähe dieser Nullstellung des Apparates (Parallelität der Hauptschnitte der beiden Nicols), ist die Beurteilung geringer Intensitätsunterschiede außerordentlich schwer, ihre ge-

<sup>1)</sup> F. Grünbaum, Inaug.-Diss., Berlin 1902, und Drudes Annalen d. Physik, Bd. XII, S. 993, 1903.

naue Messung unmöglich. Deshalb wird eine bei dieser Stellung der Nicolschen Prismen gemachte Kompensation einer etwaigen Ungleichheit in den Intensitäten der beiden Gesichtsfelder für die empfindlichere Stellung der Nicols (Hauptschnitte einen Winkel von  $55^\circ$  oder mehr miteinander bildend) nicht mehr gelten können. Ferner sind alle Rauchgläser mehr oder weniger gefärbt und absorbieren verschiedene Lichtmengen bei verschiedenen Wellenlängen.<sup>1)</sup> Speziell für die Regionen, wo wir die Extinktionskoeffizienten des Oxyhämoglobins gewöhnlich messen, ist das Lichtextinktionsvermögen von Rauchgläsern verschieden. So fanden wir mit dem Apparat von König und Martens für 2 aneinander gekittete Rauchgläser bei einer mittleren Wellenlänge von  $538 \mu$   $J' = 0,113$  und bei  $560 \mu$ ,  $J' = 0,144$ . Bei dem Hüfnerschen Spektrophotometer kann das Lichtextinktionsvermögen des Rauchglaskeiles nicht direkt am Apparat gemessen werden, weil die Extinktion zu gering ist. Man kann es aber indirekt ermitteln, indem man eine stark absorbierende Lösung (z. B. Hämoglobin) vorschaltet und die Änderungen des Wertes von  $\epsilon$  bei verschiedenen Stellungen des Keiles auch bei verschiedenen Wellenlängen verfolgt. Die folgende kleine Tabelle gibt die Werte des Extinktionskoeffizienten bei unserer gewöhnlichen Versuchsanordnung, zuerst mit vollkommen ausgeschaltetem Keil und dann bei fortschreitenden Dicken des Keiles, den Keilskalentteilen 0,5 und 0,8 entsprechend.

Diese geringen Verschiebungen des Keiles, die in der sogenannten Nullstellung des Apparates einen kaum wahrnehmbaren Unterschied in den Intensitäten der beiden Gesichtsfelder hervorrufen, haben einen sehr großen Einfluß auf den Wert des Extinktionskoeffizienten, da man diesen in einer Stellung der Nicols mißt, in der der Apparat für kleine Intensitätsunterschiede sehr empfindlich ist. Das Rauchglas scheint ziemlich achromatisch zu sein; wenigstens lassen sich keine großen Unterschiede in der relativen Zunahme des Extinktionskoeffizienten für die beiden Spektralgegenden erkennen, was sofort eine

<sup>1)</sup> Vgl. Vierordt, Die Anwendung des Spektralapparates usw., Tübingen 1873, S. 12.

Änderung des Wertes  $\frac{\epsilon'}{\epsilon}$  zufolge haben würde. Die Werte für  $\frac{\epsilon'}{\epsilon}$  schwanken bloß innerhalb der Fehlergrenzen.

Einfluß des Rauchglaskeiles auf den Extinktionskoeffizienten.

Lösung	Stand des Keiles	$\epsilon$	$\epsilon'$	$\frac{\epsilon'}{\epsilon}$
I.	0,0	0,598	0,948	1,59
	0,5	0,666	1,03	1,55
	0,8	0,680	1,07	1,57
II.	0,0	0,591	0,934	1,57
	0,5	0,649	1,02	1,57
	0,8	0,672	1,04	1,55
III.	0,0	0,510	0,802	1,57
	0,5	0,564	0,883	1,57
	0,8	0,576	0,895	1,56

$\epsilon$  wurde bei der mittleren Wellenlänge von  $560 \mu\mu$  und  $\epsilon'$  bei  $538 \mu\mu$ ; Okularspaltbreite entspricht  $8,5 \mu\mu$ ; Eintrittsspalt  $1/40$  mm.

Aus diesen Gründen haben wir immer mit dem vollkommen ausgeschalteten Rauchglaskeil gearbeitet.

4. Beleuchtungsvorrichtung. Bei der Messung mit dem Hüfnerschen Apparat geht man von einer Stellung aus, in der die Intensitäten der beiden Gesichtsfelder gleich sind. Da diese Gleichheit nicht etwa durch eine Kompensation mittels der optischen Teile des Apparates erreicht werden darf, so ist für alle genauen Messungen unerläßliche Voraussetzung, daß die beiden Spalthälften gleichmäßig beleuchtet werden. Dies ist streng genommen mit der primitiven Beleuchtungsanordnung am Hüfnerschen Apparat nicht möglich. Denn der Eintrittsspalt wird immer von einem umgekehrten mehr oder weniger zerstreuten Bild des Strumpfes der Auerlampe beleuchtet, und der Strumpf glüht nicht in allen seinen Teilen gleichmäßig. Dies wird besonders auffallend und störend, wenn die Lampe nicht zentriert ist oder wenn die Gaszufuhr starken Schwankungen unterliegt. Eine vorgeschaltete Mattglasplatte hebt die

kleinen bei zentrierter Lampe und stark glühendem Strumpf vorkommenden Unregelmäßigkeiten auf.<sup>1)</sup>

Aus dem Gesagten ergibt sich, daß der relative Wert von  $\epsilon$  sehr von der Versuchsanordnung abhängig ist. Im speziellen Fall des Oxyhämoglobins ruft eine Änderung in der Breite des Eintritts- oder Okularspaltes eine ungleichsinnige Änderung in den Werten von  $\epsilon$  und  $\epsilon'$  hervor. Das hängt damit zusammen, daß  $\epsilon$  eine Anzahl von symmetrisch um ein Minimum zunehmenden einzelnen Extinktionen darstellt, während  $\epsilon'$  einer Anzahl ziemlich gleichmäßig von beiden Seiten eines Maximums abnehmender Extinktionen entspricht. Je mehr die Versuchsbedingungen sich den theoretisch verlangten (Messung bei einer einzelnen Wellenlänge) nähern, um so höher wird der Wert von  $\epsilon'$ , um so niedriger  $\epsilon$  und folglich um so höher  $\frac{\epsilon'}{\epsilon}$ . Spektrophotometrische Werte ohne Angaben über die Konstruktion des Apparates, die Eichung des Prismas in Wellenlängen, über etwaige Kompensationsvorrichtungen und über die Breite des Eintrittsspalt und der Okularblende sind, insofern sie mit anderen spektrophotometrischen Werten verglichen werden sollen, vollkommen wertlos.

## II. Gasometrischer Teil.

Bei den Versuchen, welche den Zweck hatten, das Volumen Sauerstoff, das von der Gewichtseinheit Hämoglobin gebunden wird, zu bestimmen, kam es hauptsächlich darauf an, eine zuverlässige und handliche Methode zu benutzen. Es kamen in Betracht 1. die Blutgaspumpe, 2. die Sauerstoff austreibung mittels Ferricyankalium nach Haldane bzw. die Kohlenoxyd austreibung nach Haldane und Hüfner. Die Auspumpungs-

<sup>1)</sup> Die primitive Beleuchtungsvorrichtung, die geringe zulässige Größe der Vergleichsfelder, die unpassende Form des Absorptionsgefäßes sowie die erheblichen Schwierigkeiten bei der raschen und sicheren Einstellung auf gleiche Helligkeit der beiden Gesichtsfelder haben uns dazu geführt, Abstand von dem Gebrauch des Hüfnerschen Spektrophotometers zu nehmen und weitere Versuche nur noch mit dem Apparat von König, Martens und Grünbaum anzustellen.

methode mit der Blutgaspumpe gehört sicher nicht zu den handlichsten Methoden und erfordert eine vollständige Gasanalyse. Ferner war an die Möglichkeit eines Sauerstoffverlustes während des Auspumpens durch Verbrauch für leicht oxydable Substanzen im Serum und in Blutlösungen zu denken. Schließlich muß man sich eines nicht direkt bestimmbareren Absorptionskoeffizienten für Sauerstoff in Blut bedienen. Aus diesen Gründen konnten wir nicht erwarten, richtige Zahlen für die absolute Menge des vom Hämoglobin allein gebundenen Sauerstoffs zu bekommen. Mit der Ferricyanidmethode nach Haldane haben Hüfnér und v. Zeynek<sup>1)</sup> sogar bei Anwendung größerer Mengen Blutes bzw. Hämoglobins, als die sind, mit denen Haldane gearbeitet hat, keine übereinstimmenden Zahlen bekommen können. Die Kohlenoxydaustreibungsmethode mit Ferricyanid in der von Hüfnér<sup>2)</sup> angegebenen Versuchsanordnung wäre die einfachste Methode; denn hier ist die Gefahr einer Sauerstoffzehrung durch die Anwendung des indifferenten Kohlenoxyds beseitigt; die Absorptionskoeffizienten fallen weg, da man Blutlösung und Ferricyankaliumlösung unter gleichem Druck mit Kohlenoxyd sättigt und nach Austreibung des Kohlenoxyds aus dem CO-Hämoglobin durch Zusammenbringen der beiden Lösungen das rückständige Kohlenoxyd unter dem Anfangsdruck messen kann. Mit dieser Methode erhielten wir konstante Werte bei Lösungen vom Rinderblut, nicht aber beim Menschenblut. Der Grund lag daran, daß, obwohl es allem Anschein nach gelang, das Menschenblut durch  $\frac{1}{10}$  %ige Sodalösung vollständig lackfarben zu machen, jedesmal im Augenblick des Zusammenbringens der konzentrierten Blut- und Ferricyankaliumlösung das Stroma der roten Blutkörperchen in Massen wieder ausfiel. Man erhält dann ein fast opakes, schokoladenfarbiges Gemisch. Es ist klar, daß mit dem Stroma sehr viel Hämoglobin mitgerissen wird und daß dieses nicht in Lösung befindliche Kohlenoxydhämoglobin von Ferricyankalium nicht mehr angegriffen wird. Wir erhielten

<sup>1)</sup> R. v. Zeynek, Arch. f. Anat. u. Physiol., physiol. Abtl., 1899. Hüfnér, ebenda.

<sup>2)</sup> Hüfnér, Arch. f. Anat. u. Physiol., physiol. Abtl., 1903.

immer viel niedrigere Werte als für Rinderblut.<sup>1)</sup> So fanden wir beim normalen Menschenblut eine Kohlenoxydabgabe von 1,17 ccm pro Gramm Hämoglobin und bei Polycythämie bloß 0,66 ccm CO pro Gramm Hämoglobin,<sup>2)</sup> während beide Blutarten nach der absorptiometrischen Kohlenoxydmethode Werte in der Nähe von 1,34 ccm CO pro Gramm Hämoglobin gaben (normales Blut 1,33 ccm und Polycythämie 1,32 ccm). Wie schon erwähnt, erhielten wir beim Rinderblut nach der Kohlenoxydaustreibungsmethode durch Ferricyankalium gute Werte — in einer Serie von 4 Versuchen Werte zwischen 1,32 ccm und 1,35 ccm für die Kohlenoxydabgabe.

Wir kehrten deshalb zu der im Jahre 1894 von Hüfner<sup>3)</sup> angegebenen absorptiometrischen Methode zurück. Das Prinzip der Methode ist folgendes. Eine vollständig reduzierte und entgaste Blut- oder Hämoglobinlösung wird mit einem bekannten Volumen Kohlenoxyd geschüttelt. Der Unterschied zwischen dem vor und nach dem Schütteln vorhandenen Gasvolumen stellt die von der Lösung aufgenommene Menge Kohlenoxyd dar; diese besteht aus zwei Teilen, aus einem in der Flüssigkeitsmenge dem Druck proportional einfach gelösten Teil und einem meist größeren Teil, der innerhalb gewisser Grenzen vom Druck unabhängig und von dem Hämoglobin als chemisch gebunden anzusehen ist. Um die Methode anwenden zu können, mußten wir Kenntnis davon haben, in welchen

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<sup>1)</sup> Das Verfahren von Haldane, wobei das Blut durch Ammoniak lackfarben gemacht wird, konnten wir nicht anwenden, da wir dann mit dem unbekannten Dampfdruck des Ammoniaks zu rechnen hätten. Barcroft will diesen Nachteil überwunden haben, indem er eine zweite Bestimmung mit Wasser und Ammoniak allein in einem zweiten Apparat gleichzeitig mit der eigentlichen Blutgasbestimmung ansetzt und diesen zweiten Apparat als Thermobarometer dienen läßt. Es ist aber durchaus nicht bewiesen, ist vielmehr sehr unwahrscheinlich, daß der Dampfdruck des Ammoniaks im Wassermanometer und im Blutmanometer der gleiche ist.

<sup>2)</sup> Anmerkung bei der Korrektur: In noch nicht veröffentlichten Versuchen hat G. B. Gruber an unserer Klinik bei einigen Polycythämikern eine erhöhte Resistenz der Erythrocyten gefunden; da unsere Versuche mit sehr konzentrierten Lösungen angestellt wurden, könnte es danach doch möglich sein, daß ein geringer Teil des Hämoglobins ungelöst blieb.

<sup>3)</sup> Hüfner, Arch. f. Anat. u. Physiol., physiol. Abtl., 1894.

Mengen Kohlenoxyd von einer Blut- oder Hämoglobininlösung einfach gelöst wird. Ganz allgemein weiß man, daß die Löslichkeit von Gasen in Wasser durch das Vorhandensein gelöster Substanzen erniedrigt wird.<sup>1)</sup> Die am meisten gebrauchte Einheit für die Löslichkeit von Gasen ist der Bunsensche Absorptionskoeffizient. Man versteht darunter das auf 0° und 760 mm Quecksilberdruck reduzierte Gasvolumen, das von der Volumeneinheit der Flüssigkeit unter einem Druck von 760 mm aufgenommen wird. Um die absolute Menge des vom Hämoglobin chemisch gebundenen Kohlenoxyds bestimmen zu können, mußten wir den Wert des Absorptionskoeffizienten,  $\alpha$ , bei der Versuchstemperatur und für die angewandte Hämoglobinkonzentration der Lösung kennen.

Die Bestimmung der Löslichkeit von Kohlenoxyd oder Sauerstoff in Lösungen von Hämoglobin stößt aber auf ganz besondere Schwierigkeiten. Da das Hämoglobin mit dem Kohlenoxyd bezw. Sauerstoff in eine lockere chemische Verbindung eingeht, so ist eine direkte Bestimmung des Absorptionskoeffizienten von vornherein ausgeschlossen; man kann sie nur auf indirektem Wege vornehmen. Hüfner hat in seiner Arbeit von 1894 versucht, den Absorptionskoeffizienten dadurch zu ermitteln, daß er oberhalb des Druckes, bei dem nahezu alles Hämoglobin mit CO verbunden ist, die Drucke des Gases über die Lösung änderte und aus den Volumenänderungen des Gases den Absorptionskoeffizienten berechnete. Hierbei ist zu bemerken, daß man mit außerordentlich kleinen Differenzen arbeitet und die Fehler dadurch sehr groß werden; es entspricht z. B. eine Druckdifferenz von 100 mm im Hüfnerschen Absorptiometer (größere Druckdifferenzen sind schwer zu erhalten) bei Anwendung von 205,62 ccm Blutlösung einer Volumenänderung von höchstens 0,50 ccm Kohlenoxyd. Nun geht die Genauigkeit der Messung mit dem Hüfnerschen Apparat nur bis in die zweite Dezimale. Fehler von 0,05 ccm sind zwar noch zulässig, der Fehler ist jedoch meistens größer; ein Fehler aber von 0,05 ccm bei der Volumenmessung hat schon eine Änderung von 6 % in dem Wert des berechneten Absorptionskoeffizienten zur

<sup>1)</sup> J. J. Mackenzie, Wiedemanns Annalen der Physik, Bd. I, 1877.

Folge. Dadurch allein lassen sich die bis etwa 12% betragenden Schwankungen bei den Hufnerschen Werten für  $\alpha$  erklären.

Hüfner<sup>1)</sup> hat auch die Löslichkeit von Kohlenoxyd in Methämoglobinlösungen bestimmt. Hier sind die Versuchsbedingungen viel günstiger. Kohlenoxyd und Sauerstoff werden von Methämoglobin nicht gebunden, das Molekulargewicht des Methämoglobins ist praktisch dasselbe wie das des Oxyhämoglobins, und die zu messenden Gasmengen bei der Bestimmung des Absorptionskoeffizienten in Methämoglobinlösungen sind nur einige Prozente kleiner, als die von reinem Wasser aufgenommenen Gasmengen. Praktisch aber hat diese Methode auch ihre Schwierigkeiten. Hüfner hat das Methämoglobin in der Weise hergestellt, daß er Stickoxyd in eine frische Lösung von ausgeschleuderten Blutkörperchen im offenen Gefäß eingeleitet hat, bis die Lösung braun geworden war. Bei dieser Reaktion wird auch  $\text{HNO}_3$  gebildet, und es besteht immer die Gefahr, daß das Methämoglobin weiter gespalten wird; natürlich wird auch die gebildete  $\text{HNO}_3$  den Absorptionskoeffizienten auch wieder beeinflussen. Wir haben versucht, auch unter spektrophotometrischer Kontrolle Methämoglobin auf diese Weise herzustellen und sind immer auf Schwierigkeiten gestoßen. Denn manchmal geht die Reaktion zu weit und es fällt Globin aus der Lösung heraus, und ein andermal ist die Methämoglobinbildung nicht vollständig. Wir haben schließlich die Erkennung des Endpunktes der Reaktion selbst unter Anwendung des Spektrophotometers so schwer gefunden, daß wir die Herstellung des Methämoglobins nach dieser Methode haben aufgeben müssen. Wir haben versucht, das Methämoglobin durch Einwirkung von Ferricyankalium zu erzeugen und dann die Lösung von Ferricyankalium durch Dialyse zu trennen. Die Dialyse aber nimmt einige Tage in Anspruch und es ist schwer, die Lösungen so lange, selbst bei niedriger Temperatur, bakterienfrei zu halten. Durch bakterielle Reduktion des Methämoglobins entsteht aber wieder Hämoglobin, und man bekommt darum bei der Bestimmung des Absorptionskoeffizienten zu hohe Werte.

<sup>1)</sup> Hüfner, Arch. f. Anat. u. Physiol., physiol. Abtl., 1895, S. 209.

Bohr<sup>1)</sup> umgeht alle diese Schwierigkeiten, indem er die Löslichkeit eines für Hämoglobin indifferenten Gases in Hämoglobinslösungen direkt bestimmt und hieraus sowie aus dem bekannten Absorptionskoeffizienten desselben Gases in reinem Wasser die prozentische Erniedrigung in Hämoglobinslösungen berechnet. Er erhält dann den gesuchten Absorptionskoeffizienten von Sauerstoff oder Kohlenoxyd, indem er stillschweigend annimmt, daß die Absorptionskoeffizienten aller Gase für reines Wasser die gleiche prozentische Erniedrigung durch das Hämoglobin erfahren und indem er den Absorptionskoeffizienten des betreffenden Gases für Wasser um den gleichen Bruchteil reduziert, um den der Absorptionskoeffizient des indifferenten Gases in Wasser durch das Hämoglobin erniedrigt wurde. Dieses Verfahren wäre dann berechtigt, wenn es sicher wäre, daß alle Gase die gleiche prozentische Erniedrigung ihrer Löslichkeit durch gleiche Mengen des betreffenden festen Körpers erfahren. Daß das aber nicht der Fall ist, geht mit Deutlichkeit aus einer Untersuchung von Hüfner<sup>2)</sup> über die Löslichkeit von Wasserstoff und Stickstoff in äquimolekularen Lösungen von Glukose, Alanin, Glykokoll, Acetamid und Harnstoff hervor.

	Löslichkeit von			
	Stickstoff in Wasser $\alpha = 0,0156$		Wasserstoff in Wasser $\alpha = 0,0181$	
		Prozentische Erniedrigung von $\alpha$ .		Prozentische Erniedrigung von $\alpha$ .
Glukose, 90 g in Liter	$\alpha = 0,0138$	11,5	$\alpha = 0,0166$	8,5
Alanin, äquimol. Lösung	$\alpha = 0,0121$	22,5	$\alpha = 0,0156$	13,5
Glykokoll, „ „	$\alpha = 0,0121$	22,5	$\alpha = 0,0158$	12,5
Acetamid, „ „	$\alpha = 0,0148$	5,0	$\alpha = 0,0180$	0,5
Harnstoff, „ „	$\alpha = 0,0148$	5,0	$\alpha = 0,0170$	6,0

Bei der Deutung der Bohrschen Versuche darf also nicht vergessen werden, daß ein auf diese Weise erhaltener Absorptionskoeffizient in der Berechnung seiner Versuche eingesetzt wurde.

<sup>1)</sup> Bohr, Nagels Handbuch und Skand. Archiv, 1905.

<sup>2)</sup> Hüfner, Zeitschr. f. physikal. Chemie, Bd. LVII, S. 611, 1907.

Bei der Berechnung unserer Versuche haben wir Absorptionskoeffizienten gebraucht, die auf ähnliche Weise wie die ersten Hüfnerschen, d. h., durch Volumendifferenzmessungen nach Änderung des Druckes des Kohlenoxyds in einer Blutlösung, erhalten worden sind. Obwohl wir größere Flüssigkeitsmengen (410 ccm) und die höchsten Druckdifferenzen, die mit dem Hüfnerschen Absorptionsmeter erreichbar sind, angewandt haben, sind doch die Volumendifferenzen immer noch zu klein und die einzelnen Resultate mit ziemlich großen Fehlern behaftet. Die angeführten Absorptionskoeffizienten sind Mittelwerte aus einer größeren Versuchsreihe (25); die Schwankungen der einzelnen Werte sind manchmal so groß, daß wir die Werte nicht als endgültig betrachten; wir behalten uns daher vor, die Löslichkeit von Kohlenoxyd in Blutlösungen mit einem Apparat besonderer Konstruktion zu bestimmen. Die jetzt angeführten Absorptionskoeffizienten sind für Lösungen von ausgeschleuderten roten Blutkörperchen in ausgekochtem Wasser bei 20° bestimmt. Zuerst haben wir zwei Versuche über den Absorptionskoeffizienten von Kohlenoxyd in reinem Wasser bei 20° mit unserem Apparat angestellt. Den Absorptionskoeffizienten für Kohlenoxyd in Wasser bei 20° fanden wir übereinstimmend in beiden Versuchen zu 0,0235; diese Zahl weicht nur 1% von der Winklerschen ab. Für Lösungen von ausgeschleuderten roten Blutkörperchen in ausgekochtem Wasser fanden wir bei einem Hämoglobingehalt von 1% den Absorptionskoeffizienten 0,0230; bei einem Hämoglobingehalt von 3,8% 0,0219 und bei einem 5,5%igen Hämoglobin-gehalt 0,0208.

Zum Verständnis der Resultate möge eine kurze Beschreibung der angewandten Apparate gestattet sein.

Das Absorptiometer<sup>1)</sup> besteht aus einem zweischenkeligen im großen Wasserständer montierten Manometer. Ein Schenkel ist oben durch einen Nickelaufsatz verschließbar; der andere bleibt in Kommunikation mit der Außenluft; beide kommunizieren unten miteinander und die Kommunikation kann durch einen Hahn unterbrochen und hergestellt werden. Das Schüttel-

<sup>1)</sup> Hüfner, Arch. f. Anat. u. Physiol., physiol. Abtl., 1894.

gefäß für das Blut- und Kohlenoxydgemisch besteht aus zwei Kugeln, von denen die eine mit Glasansatzrohr und verschließbarem Nickelaufsatz versehene Kugel als Behälter des Gases dient, während die andere der Blutbehälter ist. Durch die zwei Nickelaufsätze kann die Gaskugel in luftdichte Verbindung mit dem Manometer gebracht werden. Die zwei Kugeln sind durch einen Glashahn verbunden; durch einen zweiten Hahn kann die Blutkugel von der Außenluft abgeschlossen werden. Der geschlossene Manometerschenkel, die Nickelaufsätze, die Kugeln und Hahnbohrungen sind von Hüfner durch zweimaliges Auswägen mit Quecksilber kalibriert worden; diese Apparate sowie neue Kugelapparate wurden auch von uns durch doppelte Auswägungen mit Quecksilber noch einmal kalibriert.

Der Gang eines Versuches war folgender: Das defibrinierte Blut wurde 2 Stunden zentrifugiert, das Serum abgehebert und der Blutkörperchenbrei in ausgekochtem Wasser gelöst. Die wässrige Blutkörperchenlösung wurde in einen großen, dickwandigen Glasbehälter über Quecksilber eingefüllt; hier wurden 0,2—0,5 ccm einer 50%igen Lösung Hydrazinhydrat zugegeben und unter Nachströmen von Wasserstoff das Quecksilber bis auf einen kleinen Rest aus dem Behälter abgelassen. Die Blutlösung steht in dieser Weise über Quecksilber in einer Wasserstoffatmosphäre, bis das Oxyhämoglobin vollständig zum Hämoglobin reduziert ist (einige Stunden). Dann wurde das Ganze mit der Wasserstrahlpumpe verbunden und ausgepumpt, bis die Lösung beim heftigen Schütteln nicht mehr schäumt. Der evakuierte Blutbehälter und die Blutlösung wurden dann durch einen Hahn von der Außenluft abgeschlossen, die Pumpe unterbrochen und der Zweikugelapparat auf dem Blutbehälter aufgesetzt, verbunden und wiederholt mit Wasserstoff ausgewaschen und evakuiert. Schließlich wurde die Blutlösung unter Quecksilberdruck in die untere Kugel des evakuierten Zweikugelapparates übergefüllt und hier abgesperrt. Der Zweikugelapparat wurde dann von dem Blutbehälter getrennt und seine obere Kugel 3 mal alternierend evakuiert und mit Kohlenoxyd ausgewaschen und schließlich mit Kohlenoxyd gefüllt. Das Volumen des Kohlenoxyds wurde nach Aufsetzen des Apparates

auf das Manometer und Herstellung der richtigen Verbindungen an der Manometerteilung mit dem Staudingerschen Kathetometer abgelesen. Hiernach wurden alle Verbindungen wieder geschlossen, der Zweikugelapparat vom Manometer getrennt, der Verbindungshahn zwischen Blut- und Gaskugel geöffnet und das Gemisch so lange geschüttelt, bis das Volumen des Kohlenoxyds — nach wiederholtem Aufsetzen und Ablesung am Manometer beurteilt — konstant blieb. Aus dem Unterschied in dem Volumen des Kohlenoxyds vor und nach dem Schütteln mit der Blutlösung läßt sich das Volumen des von der Blutlösung aufgenommenen Kohlenoxyds unter Berücksichtigung der herrschenden Druck- und Temperaturverhältnisse leicht berechnen. Nach Abzug des einfach gelösten Kohlenoxyds von der Gesamtmenge erhält man das Volumen Kohlenoxyd, das von dem vorhandenen Hämoglobin chemisch gebunden ist.

Das Manometer wurde reichlich mit Wasser befeuchtet und die Tension des Wasserdampfes bei der Versuchstemperatur in der Berechnung eingesetzt. Sämtliche Versuche wurden in der Nähe von  $20^{\circ}$  angestellt. Es wurde immer 15—20 Minuten auf den Ausgleich der Temperatur nach jedem neuen Einsetzen des Zweikugelapparates gewartet; hierbei waren die Änderungen des Quecksilbermeniscus am Kathetometer abgelesen maßgebend. Während der Versuche war eine auf- und abgehende Rührvorrichtung, die von einem kleinen Motor getrieben wurde, am Wasserständer angebracht. Mittels dieser Vorrichtung gelang es, eine bestimmte Temperatur stundenlang ohne größere Schwankungen als  $0,2^{\circ}$  einzuhalten. Am Anfang und zwischen jedem dritten bis vierten Versuch wurde das Manometer mit aufgesetztem Zweikugelapparat unter Unterdruck und Überdruck gesetzt und dadurch auf Dichtigkeit geprüft.

Größere Vorräte von Kohlenoxyd wurden aus ameisen-saurem Natrium und  $\text{H}_2\text{SO}_4$  dargestellt, mit KOH gewaschen und über KOH in gläsernen Gasometer aufbewahrt. Etwas Pyrogallol wurde zu der unterstehenden KOH unter Luftabschluß gegeben; dabei trat nie eine Bräunung ein. Das Gas wurde in Stichproben analysiert und chemisch rein gefunden.

Um den Gang des Versuches zu verdeutlichen, sei ein vollständiges Versuchsprotokoll vom 15. VII. 08 angeführt.

### Versuchsprotokoll von 15. VII. 1908.

#### Rinderblut.

Volumen des Kohlenoxyds vor dem Schütteln mit der Blutlösung.	
Temperatur des Wassers im großen Wasserständer . . . . .	$t = 20,6^{\circ}$
Barometerstand . . . . .	$B = 736,0 \text{ mm}$
Temperatur am Barometer . . . . .	$T = 22,9^{\circ}$
Barometerstand, reduziert auf $0^{\circ}$ . . . . .	$B_0 = 733,11 \text{ mm}$
Quecksilbermaniscus, rechts (geschlossener Manometerschenkel) . . . . .	$r = 959,70 \text{ »}$
Quecksilbermaniscus, links (offener Manometerschenkel) . . . . .	$l = 959,70 \text{ »}$
Unterschied der beiden, $(r-l)$ . . . . .	$b' = 0,00$
Derselbe, reduziert auf $0^{\circ}$ . . . . .	$b'_0 = 0,00$
Quecksilbermaniscus, rechts (auf Manometerskala abgelesen) . . . . .	$m = 118,00 \text{ mm}$
Wasserdampftension bei der Versuchstemperatur . . . . .	$b'' = 18,05 \text{ »}$
Druck des Gases . . . . .	$p = 715,06 \text{ »}$
Abgelesenes Volumen, nach den Kalibriertabellen . . . . .	$v = 241,91 \text{ ccm}$
Volumen des Gases auf $0^{\circ}$ und 760 mm Quecksilberdruck reduziert . . . . .	$v_0 = 211,65 \text{ ccm}$

#### Nach dem Schütteln mit der Blutlösung.

	geschüttelt	
	1000 mal	1500 mal
Temperatur des Wassers im Ständer . . . . .	$t = 20,8^{\circ}$	$20,7^{\circ}$
Barometerstand . . . . .	$B = 736,0 \text{ mm}$	$736,4 \text{ mm}$
Temperatur am Barometer . . . . .	$T = 22,6^{\circ}$	$21,9^{\circ}$
Barometerstand, auf $0^{\circ}$ reduziert . . . . .	$B_0 = 733,16 \text{ mm}$	$733,63 \text{ mm}$
Quecksilbermaniscus, rechts (am Kathetometer abgelesen) . . . . .	$r = 983,15 \text{ »}$	$960,05 \text{ »}$
Quecksilbermaniscus, links (am Kathetometer abgelesen) . . . . .	$l = 937,20 \text{ »}$	$902,20 \text{ »}$
Unterschied der beiden $(r-l)$ . . . . .	$b' = 45,95 \text{ »}$	$57,85 \text{ »}$
Derselbe, auf $0^{\circ}$ reduziert . . . . .	$b'_0 = 45,79 \text{ »}$	$57,65 \text{ »}$
Quecksilbermaniscus, rechts (auf Manometerskala) . . . . .	$m = 94,63 \text{ »}$	$117,60 \text{ »}$
Wasserdampftension bei der Versuchstemperatur . . . . .	$b'' = 18,27 \text{ »}$	$18,16 \text{ »}$
Druck des Gases $(B_0 - b'_0 - b'')$ . . . . .	$p = 669,10 \text{ »}$	$657,82 \text{ »}$
Abgelesenes Volumen, nach Kalibriertabellen . . . . .	$v = 237,93 \text{ ccm}$	$241,84 \text{ ccm}$
Volumen des Gases auf $0^{\circ}$ und 760 mm Druck reduziert . . . . .	$v_0 = 194,65 \text{ »}$	$194,54 \text{ »}$

Bei der Hämoglobinbestimmung wurde in einer 25fach verdünnten Lösung der Extinktionskoeffizient zu 1,04 ( $\varphi = 72,50^\circ$ ) und in einer 37,5fach verdünnten Lösung zu 0,697 ( $\varphi = 63,38^\circ$ ) gefunden. Das Volumen der Lösung betrug 205,62 ccm; das Absorptionsverhältnis des Oxyhämoglobins in der untersuchten Spektralgegend ist 0,00187. Hieraus berechnet sich die Hämoglobinmenge nach der ersten Bestimmung zu 10,00 g und nach der zweiten Bestimmung zu 10,05 g, im Mittel 10,03 g. Der prozentische Hämoglobingehalt der Lösung ist 4,9%.

Das Volumen des von der Lösung aufgenommenen Kohlenoxyds ist 211,65 ccm — 194,54 ccm = 17,11 ccm; von diesem sind gemäß der Gleichung,  $V = \frac{\alpha h p}{760}$ , 3,77 ccm Kohlenoxyd physikalisch gelöst ( $\alpha = 0,0212$  für eine 5%ige Hämoglobinlösung bei  $20^\circ$ ,  $h$  bedeutet das Volumen der Blutlösung und  $p$  der Druck des Gases). Das Volumen des von 10,03 g Hämoglobin gebundenen Kohlenoxyds ist demnach 13,34 ccm; 1 g Hämoglobin bindet  $\frac{13,34}{10,03} = 1,33$  ccm Kohlenoxyd.

Eine Portion des zum Versuch verwendeten Kohlenoxyds wurde vor und nach dem Versuch analysiert.

	Volumen	Druck mm	Temperatur Grad	Reduziertes Volumen
Angewandtes Gas . . . . .	90,05	313,94	18,9	34,78
Nach Zusatz von $O_2$ . . . . .	202,23	425,96	18,3	106,22
Nach Verpuffung . . . . .	178,71	402,03	18,6	88,57
Nach Absorption der $CO_2$ (Kalikugel) . . . . .	122,79	358,55	20,4	53,91

Angewandt = 34,78 Volumenteile.

Gefunden 88,57 — 53,91 = 34,66 Volumenteile.

Nach vollendetem absorptiometrischen Versuch wurde das Gas von dem Manometer des großen Absorptiometers in ein Bunsensches Absorptionsrohr übergefüllt und analysiert.

	Volumen	Druck mm	Tempe- ratur Grad	Redu- ziertes Volumen
Angewandtes Gas . . . . .	110,27	588,67	19,7	79,5
Nach dem Stehen über Nacht mit einer Kalikugel . . .	108,26	597,04	20,1	79,5

Das Gas enthält somit keine Kohlensäure. Es wurde jetzt in ein Eudiometer übergefüllt, gemessen und mit Sauerstoff verpufft. Die gebildete Kohlensäure wurde mit einer Kalikugel absorbiert.

Angewandt wurde 60,25 Volumteile.

Gefunden 60,18 Volumteile.

Nach dem soeben als Schema wiedergegebenen Versuchsprotokoll sind die folgenden Werte gewonnen (Tab. III). Das Hämoglobin wurde jedesmal in zwei oder mehr Verdünnungen bestimmt.

Aus diesen Zahlen geht hervor, daß die Kohlenoxydaufnahme pro Gramm Hämoglobin erstens für Menschenblut und Rinderblut dieselbe ist und auch für pathologische Fälle, namentlich Polycythämie und Anämie bloß innerhalb der methodischen Fehlergrenzen, von 1,30—1,35 ccm, schwankt.

### III. Eisenbestimmungen.

Wir sind an die Eisenbestimmungen mit großer Vorsicht herangetreten, denn die Geschichte des Eisengehaltes des Hämoglobins ist sehr merkwürdig. Der Eisengehalt ist im Laufe von 30 Jahren von 0,47 auf 0,34% gesunken, ein Unterschied von rund 28%! Dieser Unterschied erklärt sich zum Teil wohl dadurch, daß Hämoglobin von verschiedenen Darstellungsverfahren und Reinheitsgrad in viel zu kleinen Mengen analysiert wurde; nahe liegt es auch, eine mangelhafte technische Ausführung der Bestimmung selbst anzunehmen. Denn in einigen Fällen wurde das Eisen in salzsaurer Lösung mit Permanganat titriert,<sup>1)</sup> und andere Untersucher hielten es für über-

<sup>1)</sup> Selbst Hüfner, Diese Zeitschrift, Bd. VII, S. 68, ist es damals

Tabelle III.

Versuch	Ver- suchs- tempe- ratur Grad	Kohlenoxyd- druck, bei dem die Blut- lösung ge- sättigt wurde mm	Vo- lumen der Blut- lösung ccm	Hämo- globin- gehalt der Lösung g	Volumen des aufge- nommenen Kohlen- oxyds ccm	Volumen des physikalisch ge- lösten Kohlen- oxyds ccm	Volumen des chemisch ge- bundenen Kohlenoxyds ccm	Kohlen- oxyd- aufnahme pro Gramm Hämoglobin ccm
Rinderblut, 20. V. 08 . . . .	20,60	599,05	409,66	18,69	31,84	6,94 ( $\alpha = 0,0215$ )	24,90	1,32
„ 26. V. 08 . . . .	20,40	654,32	409,66	14,69	27,12	7,76 ( $\alpha = 0,0220$ )	19,36	1,33
„ 15. VII. 08 . . . .	20,70	657,81	205,62	10,03	17,11	3,77 ( $\alpha = 0,0212$ )	13,34	1,33
Menschenblut vom Gesunden	20,00	670,36	205,62	9,2	16,09	3,86 ( $\alpha = 0,0213$ )	12,23	1,33
Herzkranker (R.) . . . . .	19,90	678,47	205,62	8,77	15,82	3,97 ( $\alpha = 0,0216$ )	11,85	1,35
Hemiplegie (Sch.) . . . . .	20,00	682,57	205,62	6,65	13,11	4,10 ( $\alpha = 0,0222$ )	9,01	1,35
Polycythämie I (Ulber) . . .	20,40	669,67	205,62	6,11	12,31	4,05 ( $\alpha = 0,0223$ )	8,26	1,35
„ II (Hilbringer) . . . . .	20,35	668,07	205,62	5,91	11,85	4,03 ( $\alpha = 0,0223$ )	7,82	1,32
„ III (Ziller) . . . . .	20,00	678,64	205,62	3,51	8,94	4,19 ( $\alpha = 0,0228$ )	4,75	1,35
„ IV (Kapfer) . . . . .	20,03	650,82	77,55	2,70	5,06	1,46 ( $\alpha = 0,0220$ )	3,60	1,33
Perniciöse Anämie (M.) . . .	20,10	702,58	77,55	3,21	5,85	1,56 ( $\alpha = 0,0218$ )	4,29	1,34
Chlorose II (B.) . . . . .	20,00	616,49	205,62	11,10	17,96	3,47 ( $\alpha = 0,0208$ )	14,49	1,31
„ III (E.) . . . . .	20,00	678,62	205,62	8,60	15,18	3,98 ( $\alpha = 0,0216$ )	11,20	1,30
Skorbut . . . . .	19,90	681,93	205,62	7,73	14,41	4,04 ( $\alpha = 0,0219$ )	10,37	1,34
Pseudoleukämie . . . . .	20,30	703,09	77,55	3,20	5,82	1,56 ( $\alpha = 0,0216$ )	4,26	1,33

flüssig, Doppelbestimmungen zu machen. So schreibt Bohr:<sup>1)</sup> «Die Eisenmenge ist nur in gewissen Fällen zweimal in jeder Blutprobe bestimmt worden, nämlich in einigen Fällen, in welchen die Resultate auffielen». Ferner kommt die Schwierigkeit, eisenfreie Reagenzien, insbesondere eisenfreies Zink<sup>2)</sup> zu erhalten, in Betracht. Spurenweise vorhandene Verunreinigungen, die bei gewöhnlichen Eisenbestimmungen vielfach ohne Belang sind, geben bei der Bestimmung der minimalen Eisenmengen des Hämoglobins einen sehr großen Ausschlag. Schließlich ist ein kleinerer Teil der Arbeiten über den Eisengehalt des Blutes mit dem Jollesschen «Ferrometer» gemacht worden. Diesen Arbeiten ist jeder Wert abzusprechen, nachdem Krüss<sup>3)</sup> gezeigt hat, daß bei der Rhodaneisenreaktion ein leicht zersetzliches Doppelsalz gebildet wird, dessen Farbe von dem Dissoziationsgrad abhängig ist.

Die sorgfältigsten Eisenbestimmungen verdanken wir Zinnoffsky<sup>4)</sup> im Bungeschen Laboratorium. Zinnoffsky war der erste, der mit größeren Mengen (bis 60 g) von wiederholt umkrystallisiertem Hämoglobin gearbeitet hat. Er hat sich nicht auf eine einzige Darstellungsweise beschränkt, sondern hat verschiedene Darstellungen angewandt, hat ferner das Eisen sowohl gravimetrisch wie auch volumetrisch (mit  $\text{KMnO}_4$ ) bestimmt und ist nach beiden Methoden zu gleichen Resultaten gekommen. Im Bungeschen Laboratorium wurde von Zinnoffsky und Jaquet der Eisengehalt des Hämoglobins vom Pferd, Rind, Hund und Huhn übereinstimmend zu 0,34% gefunden.

Da wir den größten Teil unserer Untersuchungen an

entgangen, daß bei der Reaktion ein Mehrverbrauch von  $\text{KMnO}_4$  stattfindet und Chlor frei wird.

<sup>1)</sup> Bohr, Skand. Archiv f. Physiol., Bd. III, S. 102.

<sup>2)</sup> 4 g eines Präparates von einer bekannten Firma, «Metallisches Zink, puriss., chem. rein granuliert, pro analysi», in eisenfreier Schwefelsäure gelöst, verbraucht 2,95 ccm Permanganat (Titer, 0,591 g Fe im Liter); 0,0017 g Fe in 4,0 g Zink = 0,04% Fe. Die Lösung gab eine intensive Rotfärbung mit Rhodankalium.

<sup>3)</sup> G. und H. Krüss, Kolorimetrie und quantitative Spektralanalyse, 1891, S. 174—183.

<sup>4)</sup> Zinnoffsky, Diese Zeitschrift, Bd. X.

Menschenblut anstellen wollten, das wir manchmal nur in kleinen Mengen bekommen konnten, kam es darauf an, eine Methode zu gebrauchen, mit der man auch bei kleinen Eisenmengen genaue Bestimmungen ausführen kann. Für die Bestimmung kleiner Eisenmengen im Blut wäre die von Neumann<sup>1)</sup> 1903 angegebene Methode ein sehr großer Fortschritt, wenn sie immer zuverlässige Resultate geben würde. Aber gegen die jodometrische Bestimmung des Eisens lassen sich viele Bedenken geltend machen. Die Reaktion, die der Methode zugrunde liegt, ist umkehrbar und man ist genötigt, ganz genau die Bedingungen einzuhalten, bei denen die Reaktion nach der einen Seite vollständig verläuft. Die Resultate sind nämlich in hohem Grad von der Acidität der Lösung und der Temperatur sowie von der Art der Titration des Endpunktes abhängig. Man braucht sich darum auch nicht zu wundern, wenn man nach den abweichenden Vorschriften von F. Mohr,<sup>2)</sup> Treadwell<sup>3)</sup> und Neumann verschiedene Resultate erhält. Nach zahlreichen Vorversuchen haben wir die Reaktion so sehr von der Temperatur und dem Salzsäuregehalt der Lösung abhängig gefunden, daß wir die jodometrische Bestimmung des Eisens mit  $1/230$ -n-Thiosulfat ganz aufgegeben haben. Dagegen ist der Vorschlag von Neumann, die Veraschung auf nassem Weg vorzunehmen, sehr wertvoll. Die Methode ist sehr bequem und rasch ausführbar. Weiter bewirkt die Erzeugung eines Niederschlages von Zinkammoniumphosphat in der ammoniakalisch gemachten Aschenlösung eine große technische Erleichterung bei der quantitativen Ausfällung des Eisens. Denn die quantitative Ausfällung des Eisens mit Ammoniak allein geht schwer und der dabei entstehende leicht flockige Niederschlag läßt sich seiner geringen Menge wegen nicht gut weiter verarbeiten. Durch Zusatz des Neumannschen Reagens und Kochen der ammoniakalisch gemachten Aschenlösung wird das Eisen in kurzer Zeit vollständig mitgefällt; der schwere Niederschlag läßt sich leicht auswaschen und weiter verarbeiten; das Filtrat gibt nach An-

<sup>1)</sup> A. Neumann, Diese Zeitschrift, Bd. XXXVII.

<sup>2)</sup> F. Mohr, Titrimethoden, 5. Aufl., 1877, S. 290.

<sup>3)</sup> Treadwell, Lehrb. d. analyt. Chemie, Bd. II, S. 521, 4. Aufl., 1907.

säuren mit HCl keine Rotfärbung mit Rhodankalium. Wir haben uns dieser zwei Vorzüge der Neumannschen Methode bedient und sind nur in der Behandlung des Niederschlages von Eisenhydroxyd und Zinkammoniumphosphat von Neumanns Vorschriften abgewichen, indem wir ihn in  $\text{H}_2\text{SO}_4$  gelöst, die Lösung mit Zink reduziert und endlich mit  $\frac{1}{100}$ -n- $\text{KMnO}_4$  titriert haben.

Das Verfahren war folgendes.

Das Hämoglobin, Blut oder eine Blutlösung wurde in einem Gemisch von konzentrierter  $\text{H}_2\text{SO}_4$  und  $\text{HNO}_3$  zu gleichen Volumenteilen nach der Neumannschen Vorschrift verascht. Die saure Aschelösung wurde verdünnt und die überschüssige Salpetrigsäure verjagt; nach dem Abkühlen wurde die verdünnte Aschelösung mit dem Zinkreagens versetzt, mit Ammoniak neutralisiert und der entstandene Niederschlag in einem kleinen Überschuß von Ammoniak gerade gelöst. Zu der Lösung wurden einige Platintetraeder, um gleichmäßiges Sieden zu erreichen, gegeben. Die Lösung wurde dann langsam bis zum Sieden erhitzt und eine halbe Stunde, eventuell länger in flottem Sieden gehalten. Die Platintetraeder verhindern gänzlich das Stoßen und Hochschleudern der Flüssigkeit, das sonst nach der Abscheidung des krystallinischen Zinkammoniumphosphats häufig auftritt. Die überstehende Flüssigkeit wurde, während sie noch heiß war, von dem Niederschlag abfiltriert und der Niederschlag 3 mal mit heißem Wasser gewaschen. Hierbei darf das Filtrat nach dem Ansäuern mit HCl keine Rhodanreaktion geben. Der Niederschlag wurde dann in verdünnter  $\text{H}_2\text{SO}_4$  gelöst, in eine große Platinschale übergeführt und mit Zink reduziert. Die vollständige Reduktion von 10 mg Eisen nimmt selbst bei Anwendung von chemisch reinem Zink, das von verdünnter  $\text{H}_2\text{SO}_4$  allein so gut wie gar nicht angegriffen wurde, in der Platinschale bloß 3 Stunden in Anspruch. Die reduzierte Lösung wurde dann durch Glaswolle, die mit  $\text{H}_2\text{SO}_4$  gewaschen war, in ein Becherglas filtriert und mit etwa  $\frac{1}{100}$ -n-Permanganat aus einer Gay-Lussacschen Bürette titriert. Das Permanganat wurde von jeder Titration gegen Thiosulfat von bekanntem Titer eingestellt. Der Titer des Thiosulfats wurde wiederholt mit  $\frac{1}{100}$ -n-Kaliumbichromat kontrolliert. Das

Kaliumbichromat war ein 3 mal umkrystallisiertes Präparat, das durch Erhitzen auf eine Temperatur, die einige Grade unter dem Schmelzpunkt lag, getrocknet worden war. (Ausführliches über die Anwendung von Kaliumbichromat als Urtitersubstanz, Zuverlässigkeit der Methode, Fehlergröße (1 pro Mille) usw., siehe Julius Wagner, Maßanalytische Studien, Habilitationsschrift, Leipzig 1898.)

Bei Vorversuchen mit Zusatz des Neumannschen Reagens zu bekannten Eisennengen stellte es sich heraus, daß das Reagens keinen Einfluß auf die Titration mit Permanganat hat; auch eine blinde Probe mit Neumannschem Reagens und wie oben weiter verarbeitet, reduziert kein Permanganat. Sämtliche angewandten Reagenzien waren eisenfrei; das Zink, das für uns von Kahlbaum hergestellt wurde, gab nach Lösung in  $\text{H}_2\text{SO}_4$  und nach langem Stehen keine Rhodanreaktion; eine Lösung von 10 g in  $\text{H}_2\text{SO}_4$  blieb nach Zusatz von 2 Tropfen  $\frac{1}{100}$ -n-Permanganat rot gefärbt.

Mit dieser Methode erhielt ich folgende Resultate.

#### I. Hämoglobinpräparate.

1,4920 g trockenes feingepulvertes Rinderhämoglobin — Rückstand von der ersten Bestimmung des Absorptionsverhältnisses — verbrauchten 8,45 ccm Permanganatlösung (Titer 0,594 g Fe in Liter).

Fe-Gehalt des Präparates  $5,02 \text{ mg} = 0,336 \%$ .

Hämoglobin, das zur zweiten Bestimmung des Absorptionsverhältnisses verwendet wurde.

Portion I 2,5594 g.

Portion II 2,8022 g.

Die Trockenbestimmung ergab einen Gehalt an trockenem Hämoglobin von 92,69% (I. 0,3625 g blieb mit 0,3360 g konstant, II. 0,4827 g wurde mit 0,4474 g konstant). Portion I, 2,3723 g trockenes Hämoglobin verbraucht 13,6 ccm Permanganatlösung (Titer, 0,586 g Fe in Liter), Portion II, 2,5973 g verbraucht 14,7 ccm Permanganat. Daraus berechnet sich:

Fe-Gehalt Portion I  $= 0,336 \%$ .

Fe-Gehalt Portion II  $= 0,332 \%$ .

Mittlerer Fe-Gehalt des Präparates  $= 0,334 \%$ .

Menschenhämoglobin, vollständig getrockneter Rückstand aus der Konstantebestimmung wog 3,3267 g, davon 6,99% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> = 0,2320 g. 3,0947 g Menschenhämoglobin verbraucht 17,2 ccm Permanganat (Titer, 0,591 g Fe im Liter). Fe im Präparat = 10,24 mg.

Fe-Gehalt des Hämoglobins = 0,331 %.

## II. Blut und Blutlösungen.

Lösung von ausgeschleuderten roten Blutkörperchen vom Gasversuch «Rinderblut, 15. VII. 08». Zwei 50 ccm-Portionen verascht. Portion I verbraucht 14,0 ccm Permanganat (Titer, 0,595 g Fe in Liter), entsprechend 8,33 mg Fe; Portion II verbraucht 14,1 ccm Permanganat = 8,68 mg Fe. Hämoglobingehalt der Lösung (spektrophotometrisch bestimmt) 4,89%.

Fe-Gehalt des Hämoglobins aus Portion I berechnet = 0,340%.

Fe-Gehalt des Hämoglobins aus Portion II berechnet = 0,342%.

Mittlerer Fe-Gehalt des Hämoglobins = 0,342%.

Rinderblut. 2 Portionen von 50 ccm. Portion I verbraucht 30,1 ccm Permanganat (Titer, 0,595 g Fe im Liter), Portion II verbraucht 30,0 ccm. Hämoglobingehalt 10,53%.

Fe-Gehalt Portion I, 17,91 mg = 0,340% Fe im Hämoglobin.

Fe-Gehalt Portion II, 17,75 mg = 0,337% Fe im Hämoglobin.

Mittlerer Fe-Gehalt des Hämoglobins = 0,339%.

## Menschenblut.

Blut von Gesunden. Portion I, 10 ccm verbraucht 7,7 ccm Permanganat (Titer, 0,595 g Fe in Liter); Portion II, 20 ccm verbraucht 15,6 ccm Permanganat. Hämoglobingehalt des Blutes 13,8%.

Fe-Gehalt Portion I, 4,58 mg = 0,332% Fe im Hämoglobin.

Fe-Gehalt Portion II, 9,28 mg = 0,336% Fe im Hämoglobin.

Mittlerer Fe-Gehalt des Hämoglobins = 0,334%.

Herzklappenfehler (R). Portion I, 20 ccm konzentrierter Lösung von ausgeschleuderten roten Blutkörperchen, 14,6 ccm Permanganat (Titer, 0,595 g Fe in Liter), Portion II, 20 ccm verbraucht 14,5 ccm. Hämoglobingehalt der Lösung 13,2%.

Fe-Gehalt Portion I, 8,69 mg = 0,329% Fe im Hämoglobin.

Fe-Gehalt Portion II, 8,63 mg = 0,327% Fe im Hämoglobin.

Mittlerer Fe-Gehalt des Hämoglobins = 0,328%.

Hemiplegie (S.). Portion I, 50 ccm konzentrierter Lösung von ausgeschleuderten roten Blutkörperchen, verbraucht 31,0 ccm Permanganat (Titer, 0,595 g in Liter). Portion II verloren. Hämoglobingehalt der Lösung 11,07%.

Fe-Gehalt Portion I, 18,46 mg = 0,334% Fe im Hämoglobin.

Polycythämie I. (U.). Portion I, 100 ccm Lösung von ausgeschleuderten roten Blutkörperchen, verbraucht 16,9 ccm Permanganat (Titer, 0,595 g Fe in Liter), Portion II, 100 ccm verbraucht 16,85 ccm Permanganat. Hämoglobingehalt der Lösung 3,0%.

Fe-Gehalt Portion I, 10,06 mg = 0,335% Fe im Hämoglobin.

Fe-Gehalt Portion II, 10,03 mg = 0,334% Fe im Hämoglobin.

Mittlerer Fe-Gehalt des Hämoglobins = 0,335%.

Polycythämie II. (H.). Portion I, 100 ccm verbraucht 16,10 ccm Permanganat (Titer, 0,595 g Fe in Liter), Portion II, 100 ccm verbraucht 16,25 ccm Permanganat. Hämoglobingehalt der Blutlösung 2,88%.

Fe-Gehalt Portion I, 9,56 mg = 0,332% Fe im Hämoglobin.

Fe-Gehalt Portion II, 9,67 mg = 0,336% Fe im Hämoglobin.

Mittlerer Fe-Gehalt des Hämoglobins = 0,334%.

Polycythämie III. (Z.). Portion I, 100 ccm Blutlösung verbraucht 10,3 ccm Permanganat (Titer, 0,595 g Fe in Liter), Portion II, 100 ccm verbraucht 10,2 ccm Permanganat. Hämoglobingehalt der Lösung 1,81%.

Fe-Gehalt Portion I, 6,13 mg = 0,338% Fe im Hämoglobin.

Fe-Gehalt Portion II, 6,07 mg = 0,335% Fe im Hämoglobin.

Mittlerer Fe-Gehalt des Hämoglobins 0,337%.

Chlorose II. Portion I, 50 ccm einer Lösung von ausgeschleuderten Blutkörperchen, verbraucht 15,5 ccm Permanganat (Titer, 0,595 g in Liter), Portion II, 50 ccm verbraucht 15,35 ccm Permanganat. Hämoglobingehalt der Blutlösung 5,41%.

Fe-Gehalt Portion I, 9,22 mg = 0,340% Fe im Hämoglobin.

Fe-Gehalt Portion II, 9,13 mg = 0,337% Fe im Hämoglobin.

Mittlerer Fe-Gehalt des Hämoglobins = 0,339%.

Chlorose III. Portion I, 100 ccm einer Lösung von ausgeschleuderten roten Blutkörperchen, verbraucht 22,9 ccm Permanganat (Titer, 0,595 im Liter), Portion II, 100 ccm verbraucht 23,0 ccm Permanganat. Hämoglobingehalt der Lösung 4,2%.

Fe-Gehalt Portion I, 13,63 mg = 0,325% Fe im Hämoglobin.

Fe-Gehalt Portion II, 13,69 mg = 0,326% Fe im Hämoglobin.

Mittlerer Fe-Gehalt des Hämoglobins = 0,326%.

Perniziöse Anämie (M). 50 ccm einer Lösung von ausgeschleuderten roten Blutkörperchen verbraucht 11,9 ccm Permanganat (Titer 0,595 g Fe im Liter). Hämoglobingehalt der Lösung 4,15%.

Fe-Gehalt der Lösung 7,08 mg = 0,342% Fe im Hämoglobin.

Skorbut (M). Portion I, 100 ccm verbraucht 20,55 ccm Permanganat (Titer, 0,595 g Fe in Liter), Portion II, 100 ccm verbraucht 20,50 ccm. Hämoglobingehalt der Blutlösung 3,76%.

Fe-Gehalt Portion I, 12,20 mg = 0,324% Fe im Hämoglobin.

Fe-Gehalt Portion II, 12,17 mg = 0,324% Fe im Hämoglobin.

Mittlerer Fe-Gehalt des Hämoglobins = 0,324%.

Pseudoleukämie. 50 ccm einer Lösung von ausgeschleuderten roten Blutkörperchen verbrauchten 11,75 ccm Permanganat (Titer, 0,595 g Fe in Liter). Hämoglobingehalt der Lösung 4,08%.

Fe-Gehalt der Lösung 6,99 mg = 0,341% Fe im Hämoglobin.

Gesamtmittel aus 16 Bestimmungen 0,335%, abgerundet = 0,34%.

#### Zusammenfassung.

Wir stellen nun zum Schluß die Werte für  $\frac{\epsilon'}{\epsilon}$ , den Eisengehalt und die Gasaufnahme des Hämoglobins bei sämtlichen Versuchen an Menschenblut in übersichtlicher Weise zusammen.

Fall	$\frac{\epsilon'}{\epsilon}$	Eisengehalt des Hämoglobins %	Kohlenoxydauf- nahme pro Gramm Hämoglobin ccm
Gesunder . . . . .	1,56	0,34	1,33
Herzkranker (R.) . . .	1,57	0,33	1,35
Hemiplegie (S.) . . .	1,57	0,33	1,35
Polycythämie I . . .	1,59	0,34	1,35
„ II . . .	1,59	0,33	1,32
„ III . . .	1,59	0,34	1,35
„ IV . . .	1,58	—	1,33
Perniciöse Anämie . .	1,58	0,34	1,34
Chlorose II . . . . .	1,60	0,34	1,31
„ III . . . . .	1,58	0,33	1,30
Skorbut . . . . .	1,58	0,32	1,34
Pseudoleukämie . . .	1,58	0,34	1,33

Aus diesen Zahlen geht mit Deutlichkeit hervor, daß die Lichtextinktion, der Eisengehalt und das Gasbindungsvermögen des Menschenhämoglobins innerhalb der methodischen Fehlergrenzen konstante

Größen sind. Diese Konstanz gilt nicht nur für das Hämoglobin des normalen Menschenblutes, sondern auch für das Menschenhämoglobin bei Polycythämien, bei perniziöser Anämie, Chlorose, Skorbut und Pseudoleukämie.

Schließlich sei es mir gestattet, Herrn Prof. Friedrich Müller für seine bereitwillige Unterstützung und sein stets entgegengebrachtes Interesse bestens zu danken. Meinem Freund, Erich Meyer, verdanke ich die Anregung zu der Arbeit und die Besorgung der Blutproben. Es ist mir eine angenehme Pflicht, Herrn Dr. E. Letsche, der nach dem Tode von Herrn Prof. Hüfner die Leitung des physiologisch-chemischen Instituts in Tübingen pro tempore übernahm, für sein Entgegenkommen, seine zahlreichen Ratschläge sowie die Anleitung und persönliche Beihilfe bei vielen Versuchen meinen verbindlichsten Dank auszusprechen.

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THE ROCKEFELLER INSTITUTE  
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INDEX

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1904-1910



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